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PRINCIPAL INVESTIGATOR: Lewis A. Chodosh, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Pennsylvania
Philadelphia, Pennsylvania 19104-3246

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Lewis A. Chodosh, M.D., Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**
University of Pennsylvania
Philadelphia, Pennsylvania 19104-3246

E-MAIL:
chodosh@mail.med.upenn.edu

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Germline mutations in the breast and ovarian cancer susceptibility gene, *BRCA1*, account for a large proportion of families with inherited breast and ovarian cancer. Interestingly, while germline *BRCA1* mutations predispose carriers to adenocarcinoma of the breast, no somatic *BRCA1* mutations have been found in sporadic primary breast cancers. This observation suggests that this molecule may normally protect the breast against carcinogenesis only during specific stages of mammary gland development. Previously, we have analyzed the temporal and spatial pattern of *Brca1* expression during normal mouse embryogenesis, in adult tissues, and during postnatal mammary gland development. These studies support a role for *Brca1* in the regulation of cell proliferation and differentiation in the breast during puberty and pregnancy. We hypothesize that *Brca1* plays a critical role in mammary gland development, and that its function is temporally restricted to particular developmental phases. During the course of this project, we have developed a novel tetracycline-inducible expression system to test this hypothesis in transgenic mice. This approach has contributed to our ability to determine how the loss or mutation of this molecule contributes to carcinogenesis in a developmental-specific manner.

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## INTRODUCTION

Genetic analysis of families in which multiple individuals have developed breast cancer suggests that 5-10% of breast cancer cases result from the inheritance of germline mutations in autosomal dominant susceptibility genes[1,2]. Germline mutations in one of these breast cancer susceptibility genes, *BRCA1*, appear to account for most families with inherited breast and ovarian cancer, and somewhat less than half of families displaying inherited breast cancer alone[3,4]. The *BRCA1* gene encodes a 220 kDa phosphoprotein that contains a RING finger motif, a transcriptional activation domain, and a BRCT domain typically found in proteins involved in cell cycle regulation and DNA damage response[4-10]. Tumors arising in patients with germline *BRCA1* mutations almost invariably display loss of the wild-type *BRCA1* allele, suggesting that *BRCA1* is a tumor suppressor gene[11]. Taken together with findings that reduction in *BRCA1* expression *in vitro* results in accelerated growth of breast and ovarian cancer cell lines, and that overexpression of *BRCA1* results in inhibited growth of such cell lines, these observations are consistent with a model in which *BRCA1* activation can result, either directly or indirectly, in the negative regulation of cellular proliferation in adult tissues[12-14]. Interestingly, however, we have found that the murine homologue of *BRCA1* is expressed at highest levels in the mouse in cellular compartments containing rapidly proliferating cells undergoing differentiation, such as are found in the breast during puberty and pregnancy[15,16]. The positive correlation between *Brca1* expression and cellular proliferation may be explained in part by the observation made in several laboratories, including our own, that the expression of this gene is regulated in a cell cycle-dependent manner with peak steady-state levels of mRNA and protein occurring just prior to and during S-phase[17-20]. The discovery that BRCA1 is phosphorylated in a cell cycle-dependent fashion, as well as the finding that *BRCA1* may be a substrate for certain cyclin-dependent kinases, suggests a possible function for *BRCA1* in the regulation of cell cycle progression and proliferation[18,21]. This hypothesis is supported by reports that *BRCA1* overexpression inhibits cell cycle progression at least in part by upregulating expression of *p21*$^{WAF1/CIP1}$, a cyclin-dependent kinase inhibitor that contributes to the growth arrest response to DNA damage[22,23]. Interestingly, studies have demonstrated that the BRCA1 protein forms a complex with Rad51[24]. Since Rad51 is required for the proper response to ionizing radiation in yeast, these studies suggest a role for BRCA1 in the response to DNA damage. The observation that BRCA1 is rapidly phosphorylated in response to DNA damage, including that caused by ionizing radiation, strongly supports this model[24,25]. It is important to note, however, that studies of tumor suppressor genes such as *Rb* and *p53* and have highlighted the fact that proteins in this class typically function in multiple pathways and processes in the cell.

Interestingly, while germline *BRCA1* mutations predispose carriers to adenocarcinoma of the breast, no somatic *BRCA1* mutations have been found in sporadic primary breast cancers. This observation suggests that this molecule may normally protect the breast against carcinogenesis only during specific stages of mammary gland development. Previously, we have analyzed the temporal and spatial pattern of *Brca1* expression during normal mouse embryogenesis, in adult tissues, and during postnatal mammary gland development. These studies support a role for *Brca1* in the regulation of cell proliferation and differentiation in the breast during puberty and pregnancy. We hypothesize that *Brca1* plays a critical role in mammary gland development, and that its function is temporally restricted to particular developmental phases. The focus of this project is to test this hypothesis by using a modified tetracycline-inducible expression system to either induce or abolish *Brca1* expression in transgenic mice during particular developmental stages in a temporally-restricted and breast-specific manner. The goal of this work is therefore to understand more clearly how the loss or mutation of this molecule contributes to carcinogenesis in a developmental-specific manner. This goal will be accomplished by pursuing the following specific aims:

## SPECIFIC AIMS

**Specific Aim 1. Develop breast-specific, tetracycline-dependent expression systems to inducibly overexpress or abolish *Brca1* expression in the mammary epithelium *in vivo*.** We will develop a modified tetracycline-inducible expression system in order to conditionally express *Brca1* or antisense *Brca1* mRNA in the breast in a temporally-restricted manner. Constructs will be generated in which the expression of the reverse tetracycline transcriptional activator, rtTA, will be breast-specific and dependent upon the presence of tetracycline. Since both the expression and function of rtTA are tetracycline-dependent, target expression constructs can be developed in which wild-type or mutant *BRCA1* cDNA clones are expressed in a tetracycline-dependent manner by appending them to *Tn10* operator-containing promoters. Additional target expression constructs will be developed in which *Brca1* antisense RNA is expressed in a tetracycline-dependent manner. Transgenic mouse lines will be generated which overexpress the reverse tetracycline transcriptional activator, rtTA, in a breast-specific manner from the mouse mammary tumor virus LTR. Transgenic mouse lines will also be generated which overexpress the reverse tetracycline transcriptional activator, rtTA, in a tetracycline-dependent manner. Finally, transgenic mouse lines will be generated which contain target DNA constructs that direct the tetracycline-dependent expression of wild-type or mutant *Brca1*, or *Brca1* antisense RNA. These transgenic strains should permit the inducible expression of target transgenes during specific stages of mammary gland development.

**Specific Aim 2. Inducibly overexpress *Brca1* in the mammary epithelium of transgenic mice during specific developmental stages.** The effect of overexpressing *Brca1* during specific stages of mammary gland development will be determined. Bitransgenic mice which express both the rtTA tetracycline-dependent transcriptional activator and a *Brca1* wild-type or mutant transgene driven by a tetracycline-dependent promoter will be derived by mating each of the transgenic strains developed in specific aim 1. *Brca1* overexpression in bitransgenic mice will be induced during specific stages of mammary gland development, including puberty, pregnancy, lactation and regression, by tetracycline treatment during the appropriate developmental window. Glands will be analyzed by morphological and molecular methods for abnormalities in mammary epithelial proliferation, differentiation and development, and for signs of hyperplasia, dysplasia and neoplasia.

**Specific Aim 3. Inducibly abolish *Brca1* expression in the mammary epithelium of transgenic mice during specific developmental stages.** The effect of abolishing *Brca1* expression during specific stages of mammary gland development will be determined by creating bitransgenic mice which express both the tetracycline-dependent transcriptional activator, rtTA, and a *Brca1* antisense transgene driven by a tetracycline-dependent promoter by mating the transgenic strains developed in specific aim 1. Reduction of *Brca1* expression in bitransgenic mice will be induced during specific stages of mammary gland development as above. Glands will be analyzed by morphological and molecular methods for abnormalities in mammary epithelial proliferation, differentiation and development, and for signs of hyperplasia, dysplasia and neoplasia.

## BODY

### Technical Objective I: Develop breast-specific, tetracycline-dependent expression systems to inducibly overexpress or abolish BRCA1 expression in mammary epithelial cells.

***Task 1:*** *Construct vectors for expressing rtTA and/or tTA in the mammary epithelium.*

In order to create an inducible expression system in mammary epithelial cells *in vivo*, we have constructed several mammary-specific and tetracycline-dependent expression vectors. Specifically, we have made use of the reverse tetracycline-controlled transcriptional activator, rtTA, that fuses the herpes simplex virus VP16 transcription activation domain with a mutant form of the DNA binding domain from the tet repressor of *E. coli*[26]. This transactivator requires tetracycline derivatives for specific DNA binding. Target genes are placed under the control of the tetO regulatory cassette from the tetracycline-resistance operon of Tn10. This system has been documented to rapidly induce gene expression in the presence of tetracycline by up to three orders of magnitude with a low level of basal expression. Moreover, the availability of numerous tetracycline analogs with varied binding affinities, as well as the use of varying concentrations of tetracycline, permits the absolute level of transgene expression to be reproducibly and precisely titrated. As such, this system is ideally suited for the tight control and rapid induction of potentially toxic genes to desired levels of expression.

Plasmid pUHD172-1neo was constructed by Gossen et al. and contains a neomycin-selectable marker as well as sequences encoding the reverse tetracycline-controlled transcriptional activator, rtTA, whose expression is driven by a constitutive CMV promoter/enhancer[26].

Plasmid pUHD15-1 was also constructed by Gossen et al. and contains a neomycin-selectable marker as well as sequences encoding the tetracycline-controlled transcriptional activator, tTA, whose expression is driven by a constitutive CMV promoter/enhancer[26].

Plasmid pMMTV.rtTA contains sequences encoding the reverse tetracycline-controlled transcriptional activator, rtTA, whose expression is driven by the mouse mammary tumor virus promoter/enhancer and was constructed by replacing the CMV promoter/enhancer of pUHD172-1neo with the entire MMTV promoter/enhancer long terminal repeat (LTR) containing 2.0 kb of upstream sequence. The MMTV LTR is widely used to obtain mammary-specific expression in transgenic mouse model systems.

Plasmid pHMG.rtTA contains sequences encoding the reverse tetracycline-controlled transcriptional activator, rtTA, whose expression is driven by the promoter of the housekeeping gene, hydroxymethylglutaryl CoA reductase (HMG CoA). This plasmid was constructed by replacing the MMTV promoter/enhancer of pMMTV.rtTA with the 2.25 kb NotI/XbaI fragment of pHMG. This fragment contains the mouse HMG promoter, the first noncoding exon, and a portion of the first intron. This construct was generated in order to attempt to direct expression to all cells in the mammary gland, inclusive of epithelial cells and stromal cells.

***Task 2:*** *Construct vectors for expressing rtTA and/or tTA in a tetracycline-dependent manner.*

Since constitutive expression of rtTA has been reported to be detrimental in some cell types, we have created a modification of the tetracycline-dependent expression system by replacing the constitutive CMV-derived promoter/enhancer driving rtTA expression in pUHD172-1neo, with the tet regulatory sequences (tetO) from Tn10, to create the autoregulatory plasmid, pTetO.rtTA (Fig. 1A). A similar approach has been successfully taken by others with the original tTA tetracycline-repressible transcriptional activator[27]. As a result, in this system the expression of the transcriptional activator, rtTA, is itself induced by the addition of tetracycline, and subsequently induces the target gene in a tetracycline-dependent manner. As a result, induction occurs at two different regulatory levels - the expression of the rtTA activator, and the binding and activation of the target promoter by rtTA.

***Task 3:*** *Construct target vectors expressing wild type and mutant forms of BRCA1.*

In order to permit the expression of target genes of interest in a tetracycline-dependent manner we have constructed a tetracycline-incucible expression vector, pTet-Target.Puro, that contains both a tetracycline-inducible promoter driving the expression of a target gene, and a puromycin-selectable marker (Fig. 1D). The puromycin-resistance gene, whose expression is driven by the PGK promoter, was obtained from the retroviral vector pLZRS as a ClaI-BspHI fragment, blunted with the Klenow fragment of DNA polymerase, and cloned in the NotI restriction site of pTet-Splice (Life Sciences).

In order to permit the inducible expression of wild-type BRCA1 in mammary epithelial cells in a tetracycline-dependent manner, a full-length cDNA encoding wild-type BRCA1 was subcloned into the HindIII site of pTet-Target.Puro to generate pTet.BRCA1.wt.

In order to permit the inducible expression of mutant forms of BRCA1 in mammary epithelial cells in a tetracycline-dependent manner, a cDNA clone encoding BRCA1 truncated at the carboxy-terminal ApaI restriction site was subcloned into the HindIII site of pTet-Target.Puro to generate pTet.BRCA1.ApaI. In addition, a cDNA clone encoding the naturally occurring mutant of BRCA1 C64G was constructed. This mutation contains a point mutation in the RING finger domain of BRCA1 that has been found to cosegregate with breast and ovarian cancer in BRCA1 families. This point mutation was created by PCR site-directed mutagenesis using overlapping PCR primers containing complementary mutational changes at C64. The resulting HindIII-BglI fragment containing the mutant region was subloned into wild-type BRCA1. The full-length HindIII fragment containing the mutant BRCA1 was then subcloned into the HindIII site of pTet-Target.Puro to generate pTet.BRCA1.C64G.

***Task 4:*** *Construct target vectors expressing BRCA1 antisense RNA.*

In order to create a system in which *Brca1* expression can be inducibly down-regulated, we have used pTet-Target.Puro to construct a tetracyline-inducible target vector, pTetO-Brca1AS, that inducibly expresses *Brca1* antisense RNA complementary to a 322 nucleotide region at the 5' end of murine *Brca1*. This region spans the putative *Brca1* translation initiation codon. A 322 bp region containing the 5' region of mouse BRCA1 was amplified by RT-PCR from first-strand breast cDNA. This region spans the translation initiation codon of BRCA1. This 322 bp PCR fragment was sequenced on both strands to verify that it contained wild-type sequence and was then subcloned into the HindIII-EcoRV site of pTet-Target.Puro in the antisense orientation to generate pTet.BRCA1.AS. As a negative control, the same 322 bp fragment was subcloned into pTet-Target.Puro in the sense orientation to generate pTet.BRCA1.S. This vector should express the same 322 nucleotide fragment in the sense orientiation.

In order to test the ability of the expression vectors described above to permit tetracycline-dependent inducible expression in mammary epithelial cells *in vivo*, we have used these constructs to generate a test inducible expression system in mammary epithelial cells *in vitro*. We have stably transfected pTetO.rtTA, which contains a neomycin-selectable marker, into HC11 mammary epithelial cells to generate the mammary epithelial cell clone, HC11.rtTA (Fig. 1A, B). This clone inducibly expresses the rtTA tetracycline-regulated transcriptional activator in the presence of tetracycline (Fig. 1B). In addition, we have demonstrated that the HC11.rtTA clone faithfully differentiates in response to the lactogenic hormones, prolactin, insulin and hydocortisone in the presence or absence of tetracycline, indicating that overexpression of rtTA does not interfere with the process of differentiation (Fig. 1C). Finally, we have demonstrated that induction of HC11.rtTA with doxycycline does not alter proliferation rates in this cell

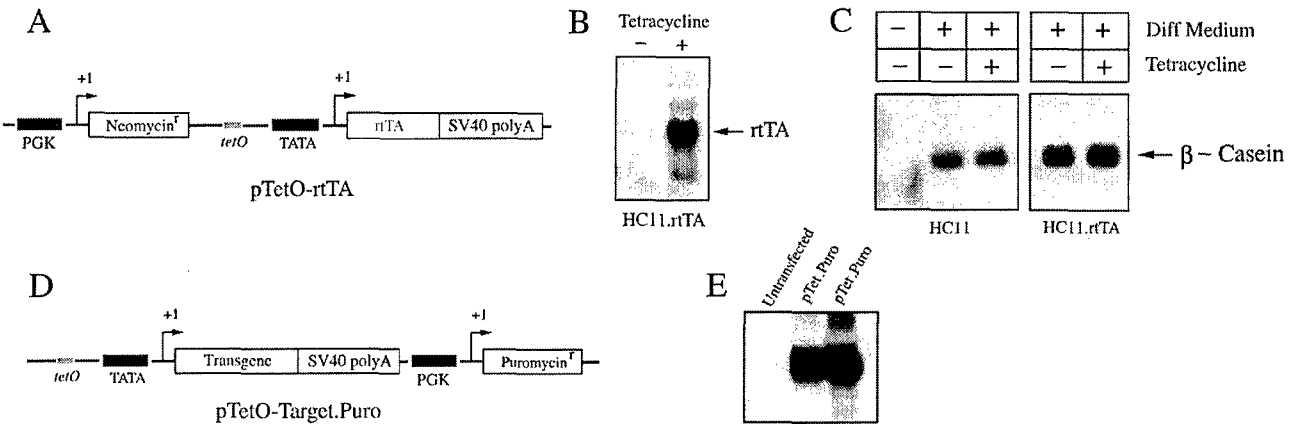


**Fig. 1: Generation of a tetracycline-inducible expression system in mammary epithelial cells**

line (not shown). In aggregate, these results demonstrate that the inducible expression of rtTA in mammary epithelial cells represents a suitable system for determining the effect of *Brca1* on mammary epithelial proliferation and differentiation.

In order to test the function of the tetracycline-incucible expression vector, pTet-Target.Puro, that contains both a tetracycline-inducible promoter driving the expression of a target gene, and a puromycin-selectable marker, pTetO-Target.Puro was stably transfected into HC11.rtTA (Fig. 1D). Puromycin-resistant clones obtained following transfection of HC11.rtTA cells with this construct express high levels of mRNA for the puromycin-resistance gene (Fig. 1E). No spontaneously puromycin-resistant clones have been detected following puromycin selection of untransfected HC11.rtTA cells.

In order to test the ability of the tetracycline-dependent transcriptional activator, rtTA, to induce the expression of the tetracycline-dependent target gene in the pTet-Target.Puro expression vector, the parental

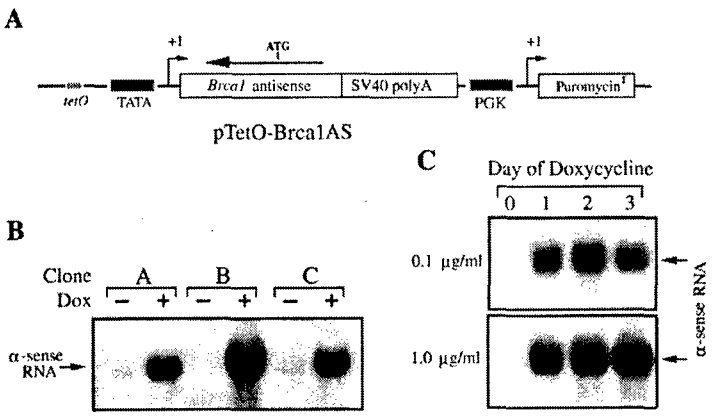


**Fig. 2: Graded inducible expression of *Brca2* antisense RNA**

HC11.rtTA cell line has been stably transfected with pTet.BRCA1.AS. Puromycin-resistant clones were demonstrated to inducibly express *Brca1* antisense RNA in the presence of doxycycline (Fig. 2B). Induction occurs rapidly, is stable, and occurs in a graded fashion dependent on doxycycline concentration (Fig. 2C). Forty one HC11 clones expressing antisense RNA were assayed for their ability to specifically

downregulate *Brca1* expression at the mRNA level. Of these, only three clones were identified on the initial screen that appeared to down-regulate *Brca1* mRNA. However, repeat analysis of these clones failed to demonstrate any significant reproducible effect on *Brca1* expression levels.

## Technical Objective II: Create transgenic mice overexpressing BRCA1 or BRCA1 antisense in the mammary epithelium.

***Task 1:*** *Create and identify transgenic lines of mice expressing rtTA and/or tTA in the mammary epithelium.*

In order to test the hypothesis that *Brca1* plays a role in the normal control of mammary epithelial proliferation and differentiation *in vivo*, this project proposed to overexpress *Brca1* in the mammary glands of transgenic mice. Given concerns regarding possible toxic effects of *Brca1* overexpression, we chose to conditionally overexpress *Brca1 in vivo* using a tetracycline regulatory system. Since no mammary-specific, tetracycline-inducible expression system existed, we decided to create and validate a novel mouse bitransgenic model system that would permit mammary-specific, tetracycline-inducible gene expression. To begin this task, we constructed a mammalian expression vector, pMMTV.rtTA, in which expression of the tetracycline-inducible transactivator, rtTA, is driven by the promoter/enhancer of the MMTV LTR. In order to generate transgenic mice harboring this construct, purified DNA containing the pMMTV-rtTA transgene was injected into fertilized oocytes harvested from superovulated FVB female mice. Two founder mice, designated MTA and MTB, were identified that harbored the transgene in tail-derived DNA and that passed this transgene to offspring in a Mendelian fashion.

Surprisingly, Northern hybridization analysis of mammary tissue from a large number of MTA transgenic female mice revealed high levels of expression of rtTA in only 50% of male and female animals harboring the MMTV-rtTA transgene. The remaining 50% of transgenic animals had no detectable rtTA expression. Intriguingly, expression of the rtTA transgene in the line of mice has been found to correlate perfectly with the methylation status of the transgene. Animals harboring an MMTV-rtTA transgene with methylation at HpaII sites near the transcription initiation site do not express rtTA in any tissue, whereas animals bearing unmethylated transgenes express rtTA at high levels. Transgenic parents of a given methylation status are able to give rise to progeny that have both methylated or unmethylated transgenes in the same litter. Thus, this phenomenon is not due to a a classical imprinting mechanism. The nature of this effect is currently being investigated further. Regardless, the fact that MMTV-rtTA transgene-positive animals frequently do not express rtTA, makes the MTA line unsuitable for further studies in the inducible expression of *BRCA1*.

Characterization of rtTA expression in the MTB line was then undertaken. Northern hybrization analysis of mammary tissue from four MTB transgenic female mice and two FVB wild-type controls, revealed high levels of expression of rtTA in all four transgenic female animals (Fig. 3). Expression levels were essentially identical in all four animals. As expected, rtTA expression was not affected by treatment of MTB animals with doxycycline. Unlike the MTA founder line, no transgene-positive animals in the MTB line have been detected that do not express the MMTV-rtTA transgene in mammary tissue. This line was therefore selected for further characterization.

Northern hybridization analysis of 13 tissues derived from male and female progeny of the MTB line of transgenic mice revealed high levels of breast-specific rtTA expression in female virgin animals (Fig. 4A). Significant levels of rtTA expression were not detected in other tissues of female mice tested, including brain, skeletal muscle, heart, lung, spleen, kidney, liver, uterus, ovary and testis. rtTA was expressed at low levels in the seminal vesicles of male mice. These results indicated that rtTA is expressed at high levels and in a breast-specific manner in MTB transgenic mice.

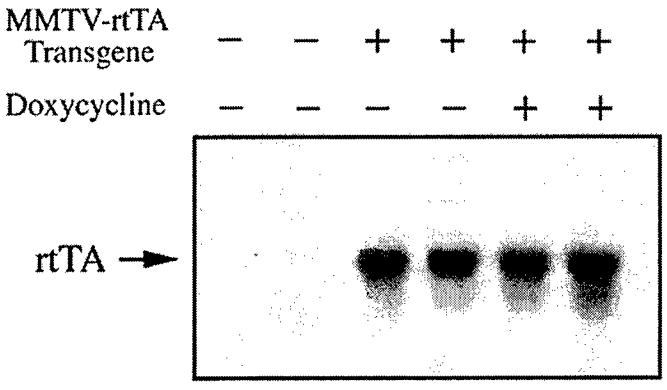


**Fig. 3: rtTA expression in MTB transgenic mice**

We have also constructed mammalian expression vectors, pTetO-LacZ, in which expression of the *LacZ* gene is driven by the tetO-containing promoter cassette, in order to serve as an indicator strain to permit the quantitative characterization of the rtTA/tetO-target bitransgenic expression system created in this proposal. This construct should permit the inducible expression of *LacZ* in response to tetracycline in cells expressing the rtTA transcriptional activator. Using an approach similar to that described above this

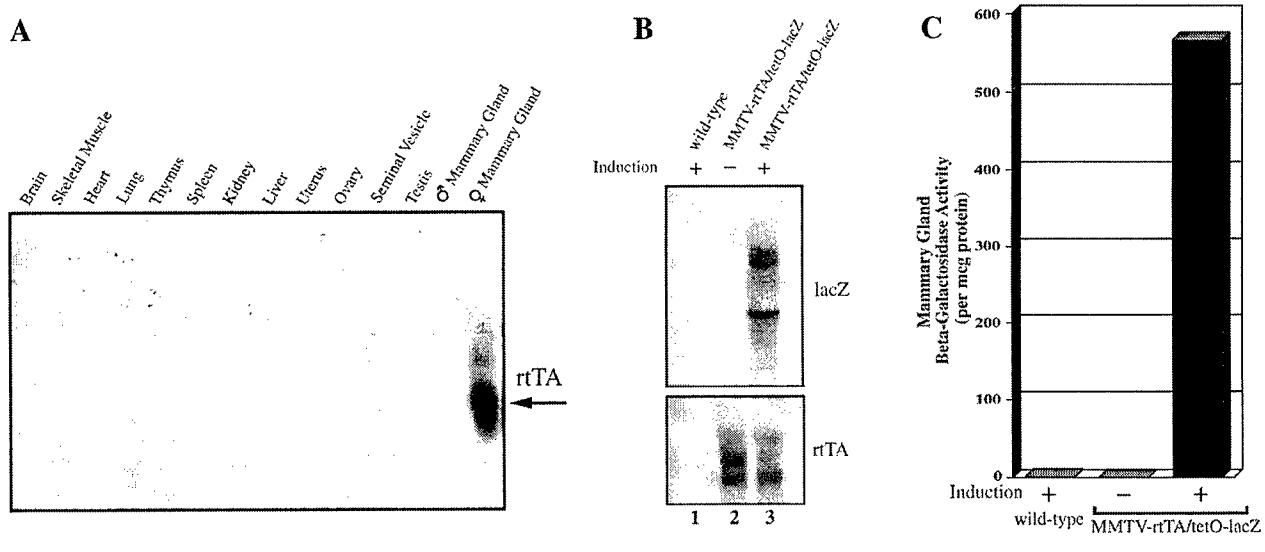


**Fig. 4: Bitransgenic system for inducibly overexpressing genes in the breast of transgenic mice**

construct, which also contains SV40 splicing and polyadenylation signals, has been used to generate transgenic mice in the FVB background. Founder animals have been identified that pass the pTetO-LacZ transgene to their offspring in a Mendelian fashion.

In order to determine whether this transgenic system will permit inducible transgene expression in the breast in response to tetracycline analogs, MMTV-rtTA transgenic mice were mated to TetO-LacZ mice and bitransgenic mice were identified. Wild-type (non-transgenic) and bitransgenic mice were treated with doxycycline. Breast tissue from wild-type and bitransgenic mice was harvested after 48 hours of treatment with doxycycline, as was breast tissue from a bitransgenic littermate that had not been treated with doxycycline. RNA was prepared from these three tissue samples and steady-state levels of *rtTA* and *LacZ* mRNA expression were assessed by Northern hybridization (Fig. 4B). As expected, bitransgenic animals expressed *rtTA* at similar levels in the presence and absence of doxycycline, whereas the *LacZ* target mRNA was only expressed in bitransgenic animals treated with doxycycline. No *LacZ* mRNA was detected either in wild-type animals, or in untreated bitransgenic animals.

In order to quantitate the level of lacZ protein expression in the induced and uninduced states, beta-galactosidase assays were performed on protein extracts made from breast tissues harvested from wild-type, MTB, TZA and MTB/TZA mice (Figs. 4C and 5). As expected, abundant beta-galactosidase activity was present in extracts prepared from bitransgenic animals treated with doxycycline for 48 hours.

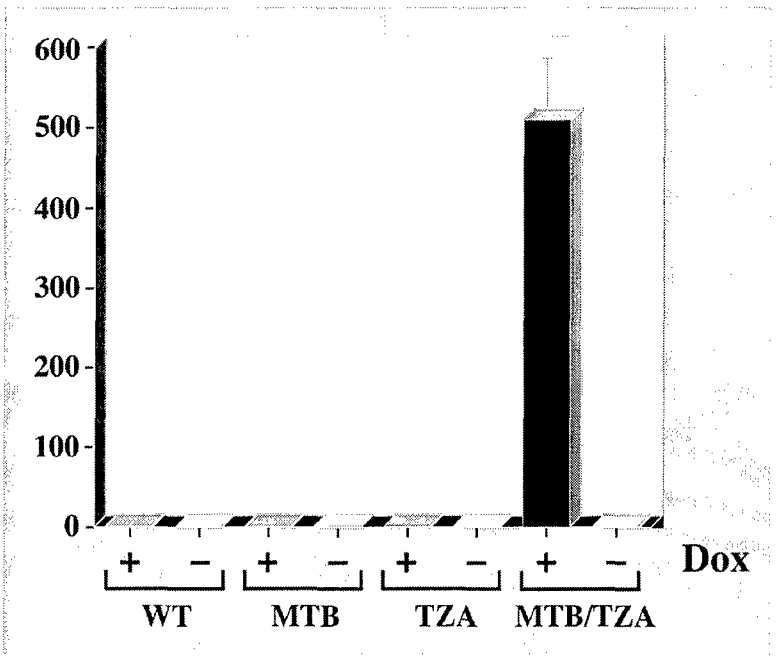


**Fig. 5A: Genotype-dependence for inducible overexpression of LacZ in bitransgenic mice**

Impressively, no beta-galactosidase activity was detected in extracts prepared from breast tissue harvested from non-transgenic, MTB, or TZA mice in the presence or absence of treatment with doxycycline. In addition, no beta-galactosidase activity was detected in protein extracts prepared from untreated bitransgenic animals. Based on the lower limits of detection for this assay, we estimate that the minimum induction of transgene expression observed in this experiment is at least 700-fold, and that the actual level of induction may be 1 or 2 logs higher. Our estimates of the minimum level of induction are comparable to that observed in a related system making use of the tTA tetracycline-dependent repressor, rather than the rtTA tetracycline-dependent transactivator[28].

Assays to detect luciferase gene expression are more sensitive and quantitative over a larger dynamic range than assays for Beta-galactosidase activity. Therefore, transgenic mice carrying the luciferase gene

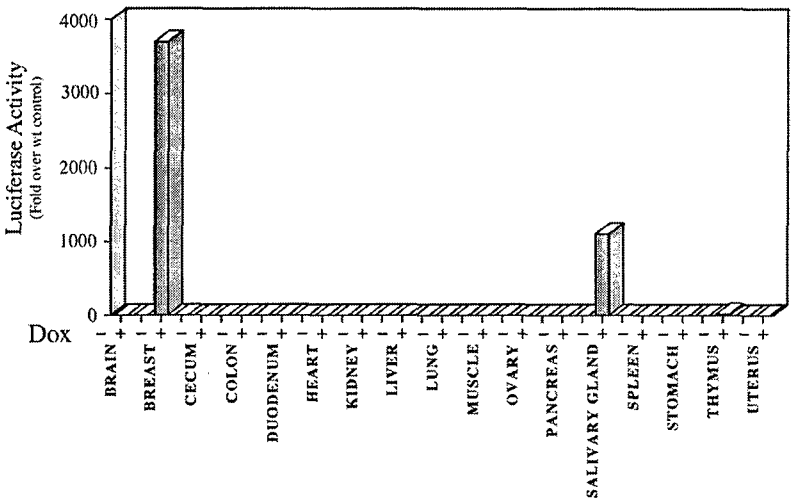


**Fig. 5B: Tissue-specificity of transgene induction in bitransgenic mice**

under the control of tet-operator sequences (TetO-Luc) were generated and crossed to MTB mice. Bitransgenic MTB/TetO-Luc mice were identified and induced for 72 hours with 2 mg/ml doxycycline. Both genetic and uninduced controls were analyzed in parallel. Luciferase assays were performed on protein samples from a panel of 17 tissues and normalized to protein concentration. These studies demonstrate the induction of luciferase activity by more than three orders of magnitude in the mammary glands of of bitransgenic mice, and somewhat lower levels of induction in the salivary glands of the animals (Fig. 2B). Low but detectable levels of expression were observed in the thymus, a tissue previously shown

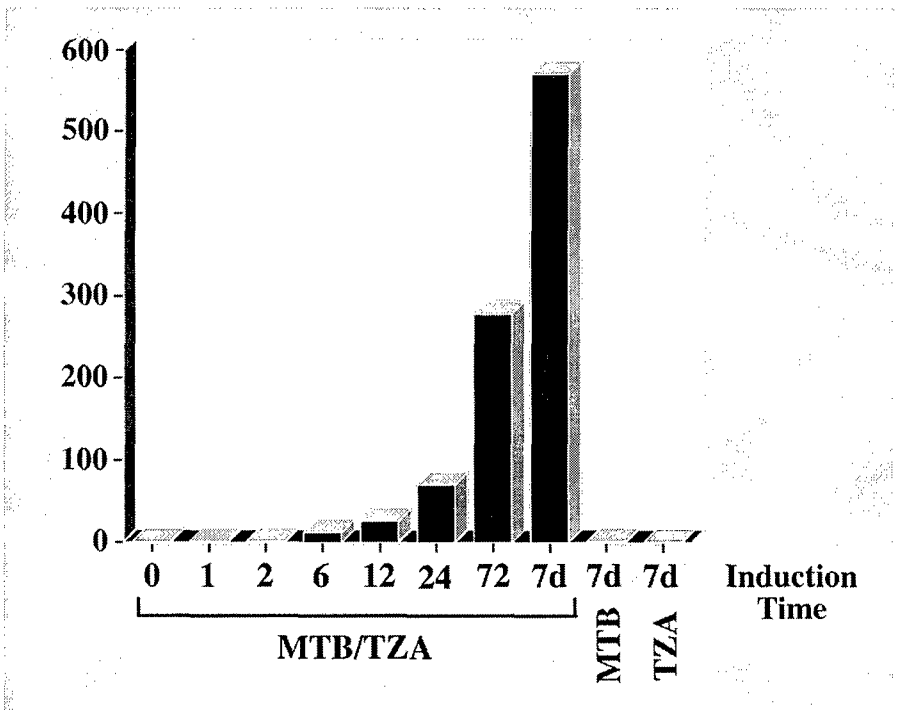


**Fig. 6: Time-course of induction of LacZ expresssion in bitransgenic mice**

to express MMTV-driven transgenes. Notably, neither mammary gland, salivary gland, nor thymus demonstrated any detectable luciferase activity in the absence of doxycycline induction (Fig. 5B). These findings confirm that this bitransgenic system is both mammary-specific and tightly regulated by doxycycline.

In order to further characterize this system, the time-course for transgene induction by doxycycline was determined (Fig. 6). Breast tissue from MTB/TZA bitransgenic mice treated with 2 mg/ml doxycycline for the indicated periods of time was harvested and protein extracts prepared. MTB and TZA transgenic animals treated with doxycycline for 7 days were used as negative controls. Beta-galactosidase assays revealed that transgene expression could be detected beginning at 6 hours following treatment with doxycycline. This initial increase represented at least a ten-fold induction of beta-galactosidase activity. Transgene expression continued to increase over the 7 day course of the experiment. No beta-galactosidase activity was detected in MTB or TZA control glands harvested from animals treated with doxycycline for 7 days.

Additional experiments performed to characterize this inducible model system included the determination of the doxycycline concentration-dependence of transgene induction (Fig. 7). MTB/TZA bitransgenic mice were treated for 72 hourse with doxycycline concentrations ranging from 0 to 8 mg/ml. Mammary tissue was subsequently harvested and protein extracts prepared. MTB and TZA transgenic animals treated with 8.0 mg/ml doxycycline for 72 hours were used as negative controls. Beta-galactosidase assays revealed that transgene expression could be detected in animals treated with as little as 0.03 mg/ml doxycycline. Treatment of bitransgenic animals with this dose of doxycycline resulted in a ten-fold induction of beta-galactosidase activity. Transgene expression increased as a function of doxycycline concentration and plateaued at 0.5 mg/ml. No beta-galactosidase activity was detected in MTB or TZA control glands harvested from animals treated with 8.0 mg/ml doxycycline.

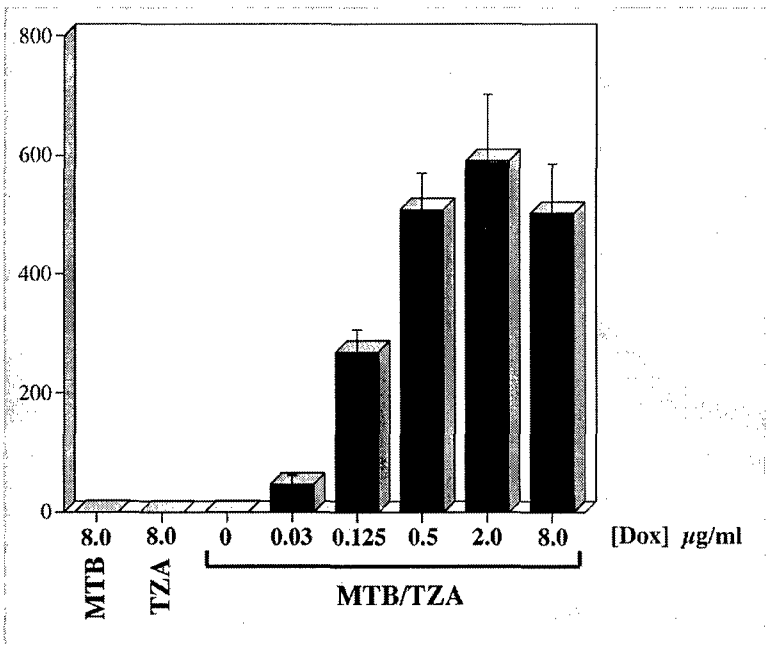


**Fig. 7: Doxycycline concentration dependence of LacZ induction in bitransgenic mice**

In order to confirm these results and to identify the cell types in the mammary gland in which inducible expression occurred, MMTV-rtTA transgenic mice were mated to TetO-LacZ mice and bitransgenic mice

were identified. Bitransgenic mice were treated with doxycycline and breast tissue was harvested after 48 hours, as was breast tissue from a bitransgenic littermate that had not been treated with doxycycline. Both

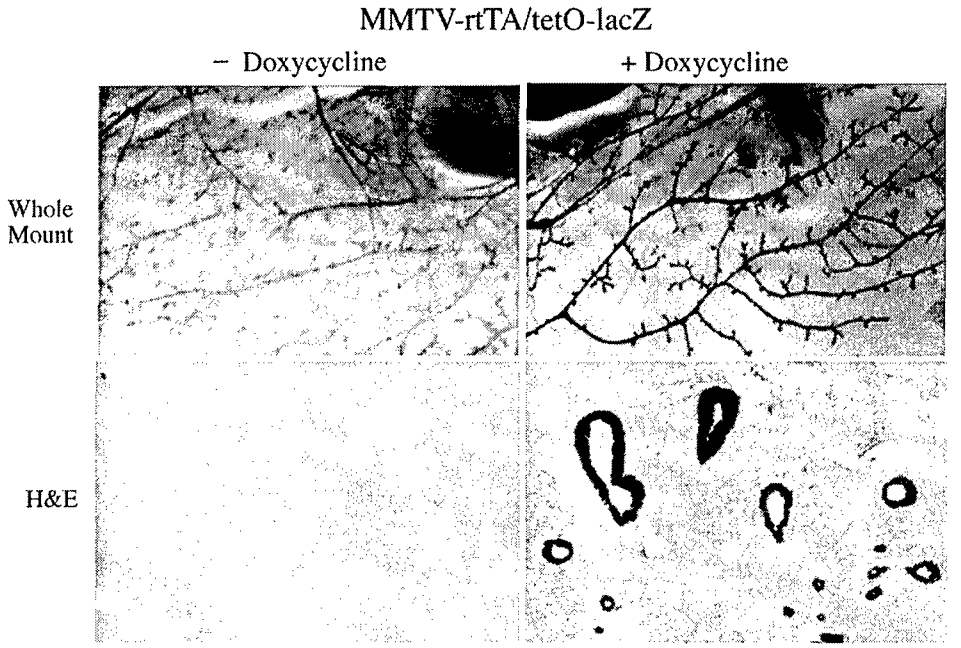


**Fig. 8: LacZ expression in the mammary epithelium of bitransgenic mice**

mammary gland whole-mounts and frozen sections from mammary glands embedded in OCT were stained *in situ* for lacZ activity (Fig. 8). Results from both whole-mounts and frozen sections demonstrated that inducible lacZ expression is confined to the mammary epithelial tree. Moreover, expression occurred at a high level and in a relatively homogeneous manner in all epithelial structures examined.

In aggregate, our results demonstrate that the MMTV-rtTA/TetO-transgene system that we have generated will permit the rapid and breast-specific induction of transgene expression at high levels, in combination with extremely low levels of expression in the uninduced state. Notably, this system permits the modulation of transgene expression levels both by varying the concentration of doxycycline and by varying the tetracycline derivative used. These properties are ideal for determining the effects of a potentially toxic transgene on specific stages of mammary gland development, and for obtaining levels of transgene expression that are in the physiological range.

In order to create transgenic lines of mice that express rtTA constitutively in all cell types in the mammary gland, including epithelial cells and stromal cells, the pHMG.rtTA transgene was injected into fertilized oocytes harvested from superovulated FVB female mice. Two founder mice, designated HTA and HTB, were identified that harbored the transgene in tail-derived DNA and that passed this transgene to offspring in a Mendelian fashion. In order to determine whether this transgenic system will permit inducible transgene expression in the breast in response to tetracycline analogs, HTA and HTB transgenic mice were mated to TetO-LacZ mice and bitransgenic mice were identified. Wild-type (non-transgenic) and bitransgenic mice were treated with doxycycline. Breast tissue from wild-type and bitransgenic mice was harvested after 48 hours of treatment with doxycycline, as was breast tissue from a bitransgenic littermate that had not been treated with doxycycline. Beta-galactosidase assays were performed on protein extracts prepared from each of these harvested breast tissues. No beta-galactosidase activity was detected

in bitrasngenic HMG.rtTA/tetO-LacZ mammary tissue from animals induced with doxycycline. We conclude that the HTA and HTB lines of mice are not be suitable for expressing inducibly target transgenes in the mammary gland.

In order to create transgenic lines of mice that express rtTA in a tetracycline-dependent manner, purified DNA containing the pTetO.rtTA transgene was injected into fertilized oocytes harvested from superovulated FVB female mice. Two founder mice, designated TTB and TTE, were identified that harbored the transgene in tail-derived DNA and that passed this transgene to offspring in a Mendelian fashion. Northern hybridization analysis of tissues derived from progeny of the TTB and TTE lines of mice failed to reveal detectable levels of rtTA expression in the mammary glands of female virgin animals (not shown). Low levels of expression of rtTA were detected in TTB and TTE animals in the thymus, kidney and spleen. In order to confirm these results, the TetO.rtTA transgenic lines of mice, TTB and TTE, were mated to TetO-LacZ mice and bitransgenic tetO.rtTA/tetO-LacZ mice were identified. Wild-type and bitransgenic mice were treated with doxycycline. Breast tissue from wild-type and bitransgenic mice was harvested after 48 hours of treatment with doxycycline, as was breast tissue from a bitransgenic littermate that had not been treated with doxycycline. Beta-galactosidase assays were performed on protein extracts prepared from each of these harvested breast tissues. No beta-galactosidase activity was detected in bitrasngenic tetO.rtTA/tetO-LacZ mammary tissue from animals induced with doxycycline. We conclude that the TTB and TTE lines of mice will not be suitable for inducibly expressing target transgenes in the mammary gland.

***Task 2:*** *Create and identify transgenic lines of mice expressing wild type and mutant forms of BRCA1 in a tetracycline-dependent manner.*

As described above, we have constructed a mammalian expression vector, pTetO.BRCA1, in which expression of *BRCA1* is driven by the tetO-containing promoter cassette. This constructs is intended to permit the inducible expression of *BRCA1* in response to tetracycline in cells expressing the rtTA transcriptional activator. Using an approach similar to that described above this construct, which also contains SV40 splicing and polyadenylation signals, has been used to generate transgenic mice in the FVB background. Initially, we identified two founder animals, TB1A and TB1B, that passed the pTetO-BRCA1 transgene to offspring in a Mendelian fashion. In order to generate more founder lines in order to increase the likelihood of identifying a TetO-BRCA1 (TB1) line of transgenic animals that is able to inducibly express BRCA1 in response to doxycycline, we have reinjected purified pTetO.BRCA1 DNA into fertilized oocytes from superovulated FVB mice. This has resulted in the generation of an additional 7 founder animals that contain this transgene in their tail-derived DNA. These additional lines of mice have been bred to wild-type FVB mice in order to determine whether they pass this transgene to their offspring in a Mendelian fashion. In order to identify lines of TB1 mice capable of inducibly expressing BRCA1, TB1 transgenic lines displaying Mendelian inheritance were mated to MTB transgenic mice, and the resulting MTB/TB1 bitransgenic mice were analyzed for BRCA1 transgene expression in the presence of doxycycline (Fig. 9). This analysis resulted in the identification of two TB1 founder lines that inducibly express BRCA1 in the presence of an MTB transgene and in the presence of doxycycline induction. BRCA1 transgene induction was seen within 8 hours of induction. As expected, no induction was seen in TB1 or MTB transgenic animals treated with doxycycline. Lower levels of BRCA1 transgene expression were observed following either 24 hours or 72 hours of induction with doxycycline. It is not known whether this reflects animal to animal variation in inducibility, or whether high levels of BRCA1 induction result in apoptosis of expressing cells. In order to begin to address the question of whether the *BRCA1* that

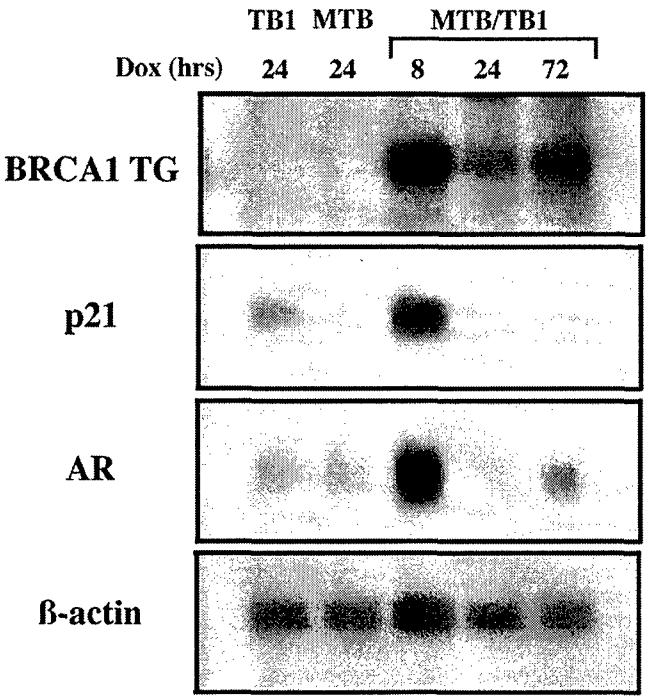


**Fig. 9: Inducible BRCA1 expression in MTB/TB1 bitransgenic mice**

is expressed is functional, Northern blots were probed with cDNA probes for p21/WAF1/CIP1 and amphiregulin. Each of these genes has previously been reported to be upregulated by BRCA1. Our initial analysis indicated that both p21 and amphiregulin are upregulated in MTB/TB1 bitransgenic mice treated with doxycycline for 8 hours. This suggested that we had succeeded in generating a bitransgenic system that is capable of inducibly expressing function BRCA1 protein in the murine mammary gland *in vivo*. Unfortunately, attempts to reproduce this result with additional MTB/TB1 bitransgenic mice, as well as TB1 and MTB control mice, have been unsuccessful. Specifically, when additional animals have been analyzed the induction of BRCA1 observed in MTB/TB1 mice has been less marked, and MTB and TB1 control mice also appear to induce BRCA1 expression following 8 hours of treatment with doxycycline (the original control samples had consisted of MTB and TB1 control mice treated with doxycycline for 24 hours (Fig. 9). Moreover, the alterations initially seen in amphiregulin and p21 expression have also been less marked, and have also been observed in MTB and TB1 control mice.

In order to extend these findings, we attempted to detect inducible expression of BRCA1 at the protein level. Protein extracts were prepared from mammary glands harvested from MTB/TB1, MTB and TB1 mice treated with doxycycline for either 8 hours or 24 hours. Immunoblotting performed on these extracts with anti-BRCA1 antibodies failed to reveal detectable expression of the BRCA1 transgene in the induced state. In an attempt to increase the sensitivity of this assay, immunoblotting was performed on mammary extracts from which BRCA1 had been immunoprecipitated. This also failed to reveal detectable BRCA1 transgene expression. We reasoned that it was possible that only a small number of cells were expressing BRCA1, and that this might impede the detection of transgene expression in mammary gland homogenates. To address this possibility, immunohistochemistry was performed on induced and uninduced MTB/TB1 mammary glands using an anti-BRCA1 antibody. No specific staining was observed. We conclude that although the two lines of TB1 mice that have been characterized inducibly express mRNA from the BRCA1 transgene, these lines either do not express functional BRCA1 or do not express functional BRCA1 in amounts sufficient to study in this system.

***Task 3:*** *Create and identify transgenic lines of mice expressing BRCA1 antisense RNA in a tetracycline-dependent manner.*

Before creating transgenic mice expressing *BRCA1* antisense RNA in a tetracycline-dependent manner, we wished to determine whether the pTetO-BRCA1.AS construct generated above would be capable of down-regulating *BRCA1* expression. In order to accomplish this, the antisense constructs were first tested for their ability to specifically reduce the expression of BRCA1 protein using the HC11 *in vitro* test system that we have generated. Achieving this goal requires the specific detection of mouse Brca1 protein. We tested several commercially available antisera directed against mouse Brca1. In our hands, none of these antisera were capable of specifically detecting mouse Brca1. As a result, we chose to generate our own anti-mouse Brca1 antisera.

GST fusion proteins from four regions of the mouse Brca1 protein were generated and expressed in E. coli. GST fusion proteins were cleaved, purified and each has been injected into two rabbits. The resulting

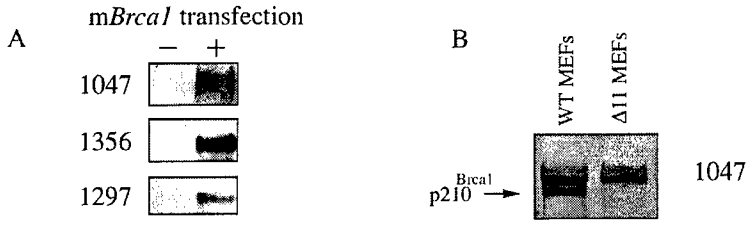


**Fig. 10: Characterization of antisera recognizing Brca1**

antisera have been tested for their ability to specifically recognize the mouse Brca1 protein. Immunoblotting analysis of HCll murine mammary epithelial cell extracts using murine Brca1 antibodies 1047, 1356, 1297 and 1226 , identified a specific band that migrated at a predicted molecular weight of 210 kDa not recognized by preimmune sera (data not shown). To confirm that the antibodies recognize bona fide mouse Brca1, 293T cells were transfected with a mouse *Brca1* cDNA and lysates were prepared for immunoblotting. These studies revealed that each of these polyclonal antibodies recognizes a specific band at the predicted molecular weight for mouse Brca1 in extracts of *Brca1*-transfected 293T cells that were subjected to immunoblotting (Fig. 10A and data not shown). To determine if Ab1047 could specifically recognize endogenous Brca1, extracts from wild-type mouse embryo fibroblasts (MEFs) and from MEFs derived from mice harboring a germline deletion of the exon 11 region of Brca1 were analyzed by immunoblotting. Northern analysis with a probe that encompasses nucleotides 4827-5354 predicted to recognize both the full length and exon 11-deleted Brca1 transcripts was performed. As expected, a 3.9 kB transcript was detected in *Brca1*$^{\Delta 11/\Delta 11}$ cells and a 7.2 kB transcript was detected in cells that express p210$^{Brca1}$ (data not shown). A similar analysis with a probe encompassing nucleotides 2541-3298 within exon 11 detected only the full length Brca1 transcript (data not shown). Accordingly, extracts prepared from wild type MEFs revealed the presence of p210$^{Brca1}$ whereas extracts prepared from *Brca1*$^{\Delta 11/\Delta 11}$ MEFS did not, confirming that the 210 kDa polypeptide recognized by mAb1 is indeed Brca1 (Fig. 10B).

In order to confirm the specificity of these antisera and to establish the subnuclear localization of p210$^{Brca1}$ and p92$^{Brca1}$, immunofluorescence analysis (IF) was performed (Fig. 11) since localization to nuclear foci during S phase is a cardinal feature of human BRCA1. Each of the four independent anti-

mouse Brca1 antisera revealed that $p210^{Brca1}$ localizes to nuclear foci during S phase in both mammary epithelial cell and WT MEFs. Strikingly, when similar experiments were performed on *Brca1*$^{\Delta 11/\Delta 11}$ fibroblasts using antisera directed against epitopes outside of exon 11, distinct nuclear foci were observed that were indistinguishable from those observed in HCll mammary epithelial cells and in wild type mouse

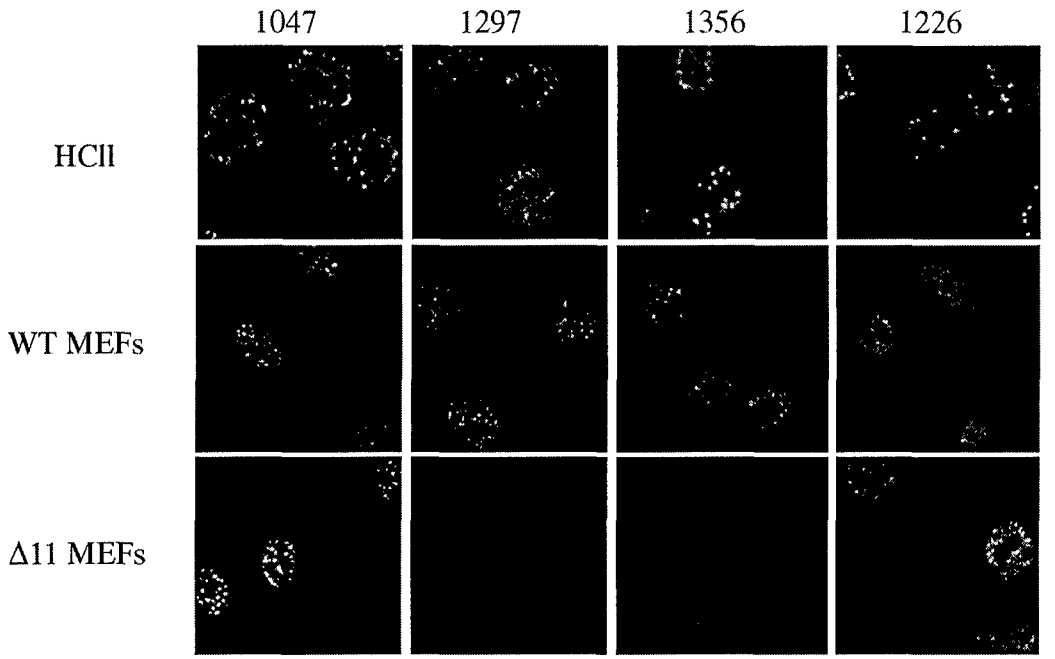


**Fig. 10: Characterization of antisera recognizing Brca1**

embryo fibroblasts (Fig. 11). Since *Brca1*$^{\Delta 11/\Delta 11}$ MEFs do not express $p210^{Brca1}$, we reasoned that any specific signal would be due to $p92^{Brca1}$. Consistent with this supposition, nuclear foci were not detected following IF using the exon 11-specific antisera, 1297 and 1356. In aggregate, these observations confirm that each of the four antisera that we have generated specifically recognize murine Brca1. At present, we believe that these are the first such antibodies described and as such represent a uniquely valuable resource.

Using the murine Brca1-specific antisera that we have generated, we screened the above HC11 clones that inducibly express BRCA1 antisense in order to determine whether the antisense fragments selected are capable of down-regulating Brca1 at the protein level. .These experiments failed to reveal any reproducible changes in Brca1 protein levels following the induction of Brca1 antisense RNA by treatment with doxycycline. Since these experiments were not successful, transgenic mice harboring this antisense construct were not generated, since they would not be expected to alter Brca1 expression levels *in vivo*.

As a parallel approach to this problem, tetracycline-inducible expression vectors were created to conditionally express four different hammerhead ribozymes designed to specifically cleave the *Brca1* transcript. Hammerhead ribozymes are catalytic RNAs that efficiently cleave RNA and thereby down-regulate gene expression. Hammerhead ribozymes can cleave any RNA containing a 5'-UH-3' consensus sequence where U can be replaced by C, and H=C, U or A. Hammerhead ribozymes have been shown to effectively and selectively inhibit gene expression in bacteria, plants, cell culture and animals, and colocalization of the ribozyme with the target RNA is critical to the efficacy of the system. We have stably transfected pTetO-Ribo1, pTetO-Ribo2, pTetO-Ribo3, and pTetO-Ribo4 into HC11 cells. Five to ten stable transformants were isolated for each construct. By RNase protection analysis we showed that each

of these cell lines has undetectable expression of the ribozyme in the uninduced state, and high levels of expression in the induced state. Clones inducibly expressing each ribozyme were subsequently tested for their ability of to inhibit Brca1 expression at the protein level using antisera directed against murine Brca1. A non-specific ribozyme was used as a negative control. Ribozymes, clones were tested after 24 or 48 hrs. exposure to doxycycline. No doxycycline-inducible reduction in Brca1 protein levels was observed in any clone or for any ribozyme.

***Task 4:*** *Breed transgenic lines of mice to create a bitransgenic line of mice expressing wild type and mutant forms of BRCA1 in the breast in a tetracycline-dependent manner.*

As described above in detail (see Task 2), MMTV-rtTA, MTB, transgenic mice characterized above were mated to offspring of the TetO-BRCA1, TB1, transgenic lines described above. Bitransgenic offspring were identified by PCR. The expression of *BRCA1* mRNA was induced in bitransgenic animals by treatment with doxycycline for increasing periods of time. Breast tissue was harvested from treated and untreated bitransgenic animals as well as control non-transgenic and monotransgenic animals. *BRCA1* expression in the breast was assayed by Northern hybridization and shown to be induced (data not shown). As described above, however, our analysis of induced MTB/TB1 bitransgenic mice indicates that BRCA1 expression is not detectable by immunoblotting, immunoprecipitation, or immunohistochemistry, or by Northern hybridization analysis of genes whose expression have been reported to be induced by BRCA1.

***Task 5:*** *Breed transgenic lines of mice to create a bitransgenic line of mice expressing BRCA1 antisense in the breast in a tetracycline-dependent manner.*

The completion of this task was deferred since we were unable to demonstrate that inducible expression of our BRCA1 antisense RNA construct was capable of down-regulating Brca1 protein expression when stably transfected into HC11 cells. Please see Task 3 for details.

**Technical Objective III: Determine the effect of inducibly overexpressing BRCA1 in the mammary epithelium of transgenic mice during specific developmental stages.**

***Task 1:*** *Analyze the phenotype of inducibly overexpressing wild type BRCA1 in the mammary epithelium during specific developmental stages.*

Although we were unsuccessful in demonstrating that we had generated bitransgenic lines of mice that express wild type forms of the BRCA1 protein in the breast in a tetracycline-dependent manner, we nevertheless performed an initial screen to determine if there was any discernible effect of inducibly overexpressing *BRCA1* mRNA on mammary epithelial development, given the possibility that low but biologically significant levels of BRCA1 protein were being expressed. Mammary glands from induced and uninduced nulliparous animals were analyzed during puberty for histology and morphology in order to identify any potential effects of BRCA1 expression after either 4 days or 21 days of induction. Examination of hematoxylin and eosin-stained sections, and carmine-stained whole mounts failed to reveal any observable defect in ductal elongation, ductal morphogenesis, terminal end bud number or terminal end bud morphology in induced bitransgenic animals.

***Task 2:*** *Analyze the phenotype of inducibly overexpressing mutant forms of BRCA1 in the mammary epithelium during specific developmental stages.*

Given our inability to demonstrate that we could generate bitransgenic lines of mice that express wild type forms of the BRCA1 protein in the breast in a tetracycline-dependent manner, and given our inability to demonstrate a tetracycline-dependent phenotype in bitransgenic lines of mice that express wild type forms of the *BRCA1* mRNA, we decided that we could not justify generating transgenic mice that inducibly express mutant forms of *BRCA1*. Therefore, this task was deferred.

**Technical Objective IV: Determine the effect of inducibly abolishing BRCA1 expression in the mammary epithelium of transgenic mice during specific developmental stages.**

***Task 1:*** *Analyze the phenotype of inducibly abolishing BRCA1 expression in the mammary epithelium during specific developmental stages.*

Given our inability to demonstrate that mammary epithelial cells that express Brca1 antisense RNA in a tetracycline-dependent manner manifest a specific reduction in Brca1 protein, and given our inability to demonstrate that mammary epithelial cells that express one of five different anti-BRCA1 ribozyme constructs, we decided that we could not justify generating transgenic mice that inducibly express either antisense *Brca1* mRNA or anti-*Brca1* ribozymes since we could not reasonably expect that these mice would display inducible reductions in BRCA1 protein levels. Therefore, this task was deferred.

## KEY RESEARCH ACCOMPLISHMENTS

- Construction of plasmid vectors for expressing rtTA in the mammary epithelium in a tetracycline-dependent or tetracycline-independent manner.
- Construction of target vectors for expressing wild-type and mutant forms of BRCA1.
- Construction of target vectors for expressing BRCA1 antisense RNA.
- Construction of target vectors for expressing BRCA1 ribozymes.
- •Generation and characterization of six transgenic lines of mice containing the TetO-rtTA, MMTV-rtTA, HMG-rtTA, TetO-LacZ, TetO-Luc, and TetO-BRCA1 expression cassettes.
- Demonstration that the MMTV-rtTA transgenic line of mice express the tetracycline-dependent reverse transcriptional activator, rtTA, at high levels, in a breast-specific manner, and in a mammary epithelial-specific manner.
- Generation of the first mammary-specific, tetracycline-inducible system for expressing transgenes in the mammary epithelium in a homogeneous manner.
- Demonstration using bitransgenic MMTV-rtTA/TetO-LacZ mice that this system permits the rapid induction of target gene expression to high levels in a breast-specific fashion in response to induction with tetracycline derivatives.
- Generation of bitransgenic mice that inducibly express BRCA1 mRNA throughout the mammary epithelium within hours following the administration of doxycycline.
- Generation of four specific rabbit anti-mouse Brca1 antibodies.

## REPORTABLE OUTCOMES

- Generation of the first mammary-specific, tetracycline-inducible system for expressing transgenes in the mammary epithelium of transgenic mice in a homogeneous manner.
- Five manuscripts published or submitted (see attached).
- Seventeen presentations at scientific meetings and conferences.
- Three published abstracts.

## CONCLUSIONS

A number of important goals have been achieved during this project. Most importantly, we have generated a novel inducible mouse model system that displays many ideal features of an inducible, mammary-specific expression system including: 1) undetectable reporter transgene expression in the uninduced state, 2) titratable induced expression levels over a broad range with relatively little variation in expression among animals of similar age and reproductive history, 3) inducible transgene expression that is spatially homogeneous in the mammary epithelium when analyzed during puberty, pregnancy, lactation or involution, 4) prompt kinetics of induction and de-induction, and 5) utilization of an inexpensive, widely available, easily administered, and essentially inert inducer. Analysis of our model system provides the first demonstration of homogeneous, inducible transgene expression in the mammary epithelium of nulliparous mice. In addition, we have also created a variety of expression vectors that have been used to create six transgenic lines of mice containing the TetO-rtTA, MMTV-rtTA, HMG-rtTA, TetO-LacZ, TetO-Luc, and TetO-BRCA1 expression cassettes. Our experiments demonstrate that the MMTV-rtTA transgenic line of mice express the tetracycline-dependent reverse transcriptional activator, rtTA, at high levels, in a breast-specific manner, and in an epithelial-specific manner. We have also succeeded in generating bitransgenic mice that inducibly express *BRCA1* mRNA throughout the mammary epithelium within hours following the administration of doxycycline. This is the first demonstration of inducible expression of *BRCA1 in vivo,* although to date we have been unable to demonstrate that functional BRCA1 protein is expressed. The studies described above demonstrate that we have made significant progress towards the completion of the specific aims of this project. Our findings suggest that the experimental system that we have generated can be used to answer important scientific questions regarding the function of the breast cancer susceptibility gene, BRCA1, in the mammary gland.

### Publications resulting from DOD New Investigator grant DAMD17-96-1-6113:

Chodosh LA. *BRCA1* and *BRCA2* expression in normal and neoplastic cells. *Journal of Mammary Gland Biology and Neoplasia* 3:389-402, 1998.

Chodosh LA, D'Cruz CM, Gardner HP, Ha SI, Marquis ST, Rajan JV, Stairs DB, Wang JY, and Wang M. Mammary gland development, reproductive history and breast cancer risk. *Cancer Research* 59:1765s-1772s, 1999.

D'Cruz CM, Gunther EJ, Hartman J, Sintasath L, Moody SE, Boxer RB, Cox JD, Ha SI, Belka GK, Golant A, Cardiff RD and Chodosh LA. MYC induces mammary tumorigenesis by means of a preferred pathway involving spontaneous K-*ras* mutations. *Nature Medicine*, In press

Huber LJ, Yang TW, Sarksian CJ, Master SR, Deng CX and Chodosh LA. Impaired DNA damage response in cells expressing an exon 11-deleted murine Brca1 variant that localizes to nuclear foci. Submitted.

Gunther EJ, Belka GK, Wertheim GBW, Wang J, Hartman JL, Boxer RB, and Chodosh LA. A doxycycline-inducible system for transgenic analysis of mammary gland biology. Submitted.

**Presentations resulting from DOD New Investigator grant DAMD17-96-1-6113:**

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| April, 1996 | "Update on the Genetic Basis of Breast Cancer"<br>Center for Cancer Care Symposium, Sacred Heart Hospital, Allentown, PA |
| June , 1996 | "Hormones, History, and Breast Cancer Risk"<br>2nd Annual FOCUS on Women's Health Research Conference<br>University of Pennsylvania, Philadelphia, PA |
| August, 1996 | "Tumor Suppressor Genes in Mammary Epithelial Differentiation and Development"<br>Gordon Research Conference on Cancer, Newport, RI |
| March, 1997 | "Coordinate Developmental Regulation of BRCA1 and BRCA2"<br>Session Chair, Experimental Models of Breast Cancer<br>AACR Conference on Basic and Clinical Aspects of Breast Cancer, Keystone, CO |
| April, 1997 | "Role of BRCA1 and BRCA2 in Mammary Development"<br>Banbury Conference on BRCA1 Function, Cold Spring Harbor, NY |
| June, 1997 | "Role of BRCA1 and BRCA2 in Mammary Development"<br>Mammary Gland Biology Gordon Research Conference, Tilton, NH |
| June, 1997 | "Role of BRCA1 and BRCA2 in Mammary Development"<br>Mammary Gland Development and Carcinogenesis Symposium<br>National Institutes of Health, Bethesda, MD |
| September, 1997 | "Cell Cycle Regulation of BRCA1 and BRCA2"<br>Institute for Human Gene Therapy Scientific Retreat |
| October, 1997 | "Breast Cancer: The Path Ahead"<br>Jackson Laboratory, Bar Harbor, ME |
| October, 1997 | "Role of BRCA1 and BRCA2 in Mammary Development"<br>Animal Models of Human Disease, Jackson Laboratory, Bar Harbor, ME |
| May, 1998 | "Mammary Gland Development, Reproductive History and Breast Cancer Risk"<br>Harriet Tischler Memorial Lecture<br>Dana Farber Cancer Institute, Harvard Medical School, Boston, MA |
| June, 1998 | "Mammary Gland Development and Carcinogenesis: Molecules at the Crossroads"<br>General Motors Cancer Research Foundation Symposium<br>National Institutes of Health, Bethesda, MD |
| May, 1999 | "Mammary Gland Development, Reproductive History and Breast Cancer Risk"<br>University of Iowa, Iowa City, Iowa |
| October, 1999 | "Reversible Tumorigenesis by c-Myc in the Breast"<br>Modeling Human Cancer in Mice<br>The Jackson Laboratory, Bar Harbor, ME |
| May, 2000 | "Models for the Influence of Reproductive History on Breast Cancer Risk"<br>Vanderbilt University School of Medicine, Nashville, TN |
| June, 2000 | "Inducible Transgenic Models of Mammary Carcinogenesis"<br>DOD ERA of HOPE Breast Cancer Meeting. June, 2000. Atlanta, GA |
| June, 2000 | "Inducible Transgenic Models of Mammary Carcinogenesis"<br>Endocrine Society 2000, Toronto, CANADA |

**Abstracts resulting from DOD New Investigator grant DAMD17-96-1-6113:**

D'Cruz CM, Gunther EJ, Hartman J, Sintasath L, Cox J, Moody SE, Ha SI, Belka GK, Golant A, Cardiff RD, and Chodosh LA. c-MYC is required for maintenance of mammary adenocarcinomas in transgenic mice. Modeling Human Cancer in Mice. The Jackson Laboratory. October, 1999. Bar Harbor, ME

Chodosh LA, D'Cruz CM, Gunther EJ, Hartman J, Sintasath L, Cox JD Moody SE, Ha SI, Belka GK, Golant A, and Cardiff RD. c-MYC is required for maintenance of mammary adenocarcinomas in transgenic mice. DOD ERA of HOPE Breast Cancer Meeting.
June, 2000. Atlanta, GA

D'Cruz CM, Gunther EJ, Hartman J, Sintasath L, Cox JD Moody SE, Ha SI, Belka GK, Golant A, Cardiff RD, and Chodosh LA. c-MYC is required for maintenance of mammary adenocarcinomas mutations in transgenic mice. Endocrine Society 2000. June, 2000. Toronto, CANADA

**Personnel supported by DAMD17-96-1-6113:**

| | |
|---|---|
| Lewis Chodosh | PI |
| George Belka | Research Sepc |
| Blaine Keister | Research Spec |
| Keith Mintzer | Research Spec |
| Thomas Yang | Graduate Student |
| James Cox | Research Spec |
| Frank Lutterodt | Research Spec |
| Sandra Marquis | Research Spec |
| Louis Sintasath | Research Spec |
| James Wang | Research Spec |
| Judith Farrell | Temporary |

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# Expression of *BRCA1* and *BRCA2* in Normal and Neoplastic Cells

**Lewis A. Chodosh**[1,2]

Current evidence strongly supports a role for the breast cancer susceptibility genes, *BRCA1* and *BRCA2*, in both normal development and carcinogenesis. Valuable clues regarding the function of these genes have been garnered through studies of their patterns of expression. A central feature of the *in vivo* pattern of *BRCA1* and *BRCA2* expression is that each of these putative tumor suppressor genes is expressed at maximal levels in rapidly proliferating cells. This feature is consistent with *in vitro* observations that *BRCA1* and *BRCA2* are expressed in a cell cycle-dependent manner. This feature is also well illustrated during mammary gland development wherein the expression of *BRCA1* and *BRCA2* is induced in rapidly proliferating cellular compartments undergoing differentiation, such as terminal end buds during puberty and developing alveoli during pregnancy. Strikingly, the spatial and temporal patterns of *BRCA1* and *BRCA2* expression are virtually indistinguishable during embryonic development and in multiple adult tissues despite the fact that these genes are unrelated. These observations have contributed to the emerging hypothesis that these genes function in similar regulatory pathways.

**KEY WORDS:** *BRCA1; BRCA2*; gene expression; development.

## INTRODUCTION

Breast cancer is the most common malignancy diagnosed among women in United States, and is the second leading cause of cancer mortality. Despite intensive efforts aimed at improving the early detection and treatment of breast cancer, mortality from this disease has only recently begun to decline. In this setting, strategies aimed at a more thorough understanding of the underlying biology of this disease are likely to be important. The markedly elevated risk of breast cancer observed in women carrying germline mutations in *BRCA1* and *BRCA2* strongly suggests that these genes play a critical role in the regulation of mammary epithelial cell growth. Moreover, the findings that the BRCA1 protein is rapidly phosphorylated following DNA damage, and that BRCA1 and BRCA2 each interact with the recombination repair protein Rad51, have implicated these genes in the cellular response to DNA damage (1). As such, studies of *BRCA1* and *BRCA2* function will likely provide insight into the mechanisms of growth control and DNA damage response in normal mammary epithelial cells, as well as serve as a foundation for understanding how the absence or mutation of these molecules promotes carcinogenesis.

Insights into gene function have frequently been gained by studying patterns of regulation during development and carcinogenesis. In particular, inferences made from the spatial and temporal patterns of *BRCA1* and *BRCA2* expression have provided the basis for several fundamental hypotheses regarding the function of these molecules. Perhaps most striking has been the observation that *BRCA1* and *BRCA2* expression patterns in a variety of tissues are virtually superimposable. These studies, when taken together with other similarities in

[1] Department of Molecular and Cellular Engineering and Department of Medicine, Division of Endocrinology, Diabetes and Metabolism, Stellar-Chance Laboratories, Room 309A, University of Pennsylvania School of Medicine, 422 Curie Boulevard, Philadelphia, Pennsylvania 19104–6100.

[2] To whom correspondence should be addressed. e-mail: chodosh@mail.med.upenn.edu



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the structure and mutational profile of these genes, have contributed to the growing realization that BRCA1 and BRCA2 are likely to function in similar pathways.

## GENE STRUCTURE AND EXPRESSION

Although the *BRCA1* and *BRCA2* genes do not share any significant sequence homology, these genes are similar in several respects. Both *BRCA1* and *BRCA2* are poorly conserved among species. Both have a similar genomic structure with an unusually large exon 11, 3.4 kb for *BRCA1* and 5 kb for *BRCA2* (Fig. 1). The *BRCA1* gene is composed of 23 exons and is expressed as a 7.8 kb mRNA transcript (2,3). This transcript encodes a protein of 1863 amino acids with a predicted size of 206 kDa, though the protein migrates on denaturing polyacrylamide gels with an apparent molecular weight of 220 kDa (1,4–9). Both the genomic structure and organization of *BRCA1* are conserved between mouse and human (10). Murine *Brca1* is expressed as a 7.5 kb mRNA transcript that encodes a protein of 1812 amino acids (10–13). The murine protein has 58% amino acid identity (72% similarity) with the human protein, but contains domains with considerably higher degrees of conservation, including the amino-terminal RING finger and carboxy-terminal BRCT domain (10–13). Unlike human BRCA1, for which several excellent immunologic reagents now exist, few reagents have been described that specifically recognize the mouse Brca1 protein.

The *BRCA2* gene is composed of 27 exons and is expressed as an 11.5–12 kb mRNA transcript (14,15). This transcript encodes a protein of 3418 amino acids with an estimated molecular weight of 384 kDa (14,15). While this protein has been detected in mammalian cell extracts, its apparent molecular weight is difficult to estimate relative to known markers given its large size (16). Mouse *Brca2* is expressed as an 11–11.5 kb mRNA transcript that encodes a protein of 3329 amino acids (17–19). The murine protein has 59% amino acid identity (72% similarity) with the human protein (17,18,20). Rat *Brca2* encodes a protein of 3343 amino acids that is 84% identical to mouse and 58% identical to human BRCA2 (18). As with Brca1, there are several domains of the mouse and rat Brca2 proteins that are highly homologous to their human orthologue, including amino and carboxyl terminal domains as well as several of the BRC repeats in exon 11 (17,18,20).

## EXPRESSION OF SPLICE VARIANTS

Analysis of the structure of several cDNA clones described in the initial cloning of *BRCA1* implied that multiple alternatively spliced forms of *BRCA1* exist (2). Several splice variants of *BRCA1* have in fact now been described, among which the *BRCA1*-Δ9,10 and *BRCA1*-Δ11b variants are the best characterized (21, 22). Importantly, the reading frame of these alternatively spliced forms is preserved, such that each would be predicted to contain the highly conserved N-terminal RING domain and the C-terminal BRCT and transcriptional activation domains. The *BRCA1*-Δ9,10 splice variant deletes the 41 amino acids encoded by exons 9 and 10 and has been shown to be expressed at levels comparable to that of full length *BRCA1* in a variety of normal tissues and cell lines (21,22) . A second major splice variant, *BRCA1*-Δ11b, retains only the first 118 nt of exon 11 (21,22). This variant is also expressed at levels comparable to that of full length *BRCA1* in normal tissues and is generated by an alternative splicing event utilizing a donor site present at

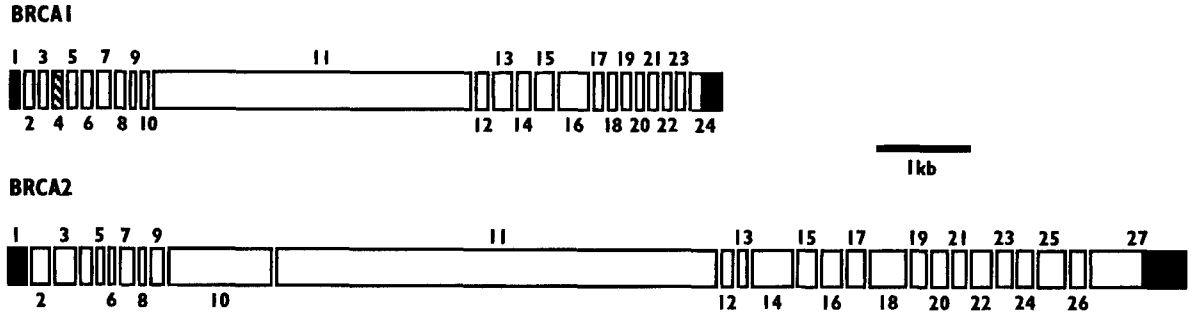


**Fig. 1.** Genomic Organization of *BRCA1* and *BRCA2*. Noncoding exons (shaded boxes) and coding exons (open boxes) of the human *BRCA1* and *BRCA2* genes are shown to scale. The alternate first exons, 1A and 1B, of *BRCA1* are not shown (see Figure 3). A nontranscribed region of *BRCA1* originally misidentified as exon 4 is indicated by crosshatching. The translational initiation codon for each gene is located in exon 2.

nucleotide positions 905–909 in exon 11. The *BRCA1*-Δ11b transcript encodes a 760 amino acid polypeptide with a predicted size of 85 kDa, though the protein migrates on denaturing polyacrylamide gels with an apparent molecular weight of 110 kDa (21). When transfected into mammalian cells, the protein encoded by the *BRCA1*-Δ11b transcript is cytoplasmic, consistent with the fact that the spliced region of exon 11 encompasses the nuclear localization signal (21). Intriguingly, although *BRCA1*-Δ11b is expressed at a level comparable to that of full-length *BRCA1* in several tissues, steady-state levels of the *BRCA1*-Δ11b message have been reported to be markedly reduced or absent in several breast and ovarian tumor cell lines (21). However, this reduced ratio of *BRCA1*-Δ11b:*BRCA1* transcripts is also observed in normal breast tissue as well as in breast tumors, suggesting that tissue-specific rather than tumor-specific splicing patterns may be operative (21). Several investigators have detected 4.4–4.6-kb mRNA species by Northern hybridization analysis of tissues and cell lines using non-exon 11-containing probes (22–24). These are likely to represent *BRCA1*-Δ11b transcripts. Moreover, this 4.4 kb message has been shown by one group to be associated with polysomes, consistent with its proposed translation (22). Exon 11b-negative mRNA transcripts are also found in the mouse during early embryogenesis as well as in embryonic stem cells (25). That this alternatively spliced form appears to have been conserved evolutionarily suggests that it may play an important physiological role.

An additional splice variant has been analyzed and shown to lack exon 11 in its entirety (nt 672–4095) (24). This variant is predicted to encode an 81 kDa protein. In vitro transcription and translation of a cDNA encoding this proposed splice variant generated a 97 kDa protein, consistent with the higher apparent as compared to calculated molecular weights of the full length BRCA1 and BRCA1-Δ11b proteins (24). Based on the observation that Northern hybridization of blots containing mRNA from human tissues using a *BRCA1*-Δ672–4095 DNA probe detects a 4.6 kb mRNA species, it has been proposed that the 4.6 kb band corresponds to *BRCA1*-Δ672–4095 (24). Although the existence of the *BRCA1*-Δ672–4095 isoform *in vivo* was confirmed by RT-PCR, as was the existence of an additional isoform, *BRCA1*-Δ789–4098, whether these transcripts are expressed at levels comparable to that of full length *BRCA1* or the *BRCA1*-Δ11b variant has not been determined (24). Given the similarity in size between the Δ672–4095, Δ789–4098 and Δ11b transcripts, as well as that of the polypeptides they are predicted to encode, the *in vivo* significance of the expression of the Δ672–4095 and Δ789–4098 isoforms remains uncertain (21,24). The existence of multiple tissue-specific splice variants of *BRCA1* has also been suggested based on the ability of Δexon11 probes to detect numerous mRNA species smaller than 7.8 kb on Northern blots containing mRNA from human tissues (24). Since few of these have been characterized in any detail or have been shown to be consistently present by independent investigators, it is unclear at present whether they represent alternatively spliced products or degradation products. Unlike *BRCA1*, multiple splice forms of *BRCA2* have yet to be described, though these clearly may exist.

## EMBRYONIC EXPRESSION

The majority of current information regarding *BRCA1* and *BRCA2* expression during embryonic development has been obtained from studies conducted in mice. Northern hybridization and RNase protection analysis demonstrate *Brca1* mRNA expression in ES cells and embryos of FVB and C57BL/6 mice at E6.5–E7.5, consistent with the onset of developmental abnormalities in *Brca1*-/-mice (19,25,26). Similarly, *in situ* hybridization analysis reveals *Brca2* mRNA expression beginning at E7.5, coincident with the onset of developmental abnormalities in *Brca2*-/-mice. The expression of each gene is markedly up-regulated from E7.5 to E13.5 and subsequently down-regulated by E18.5 (10,17,19,27,28). *Brca1* and *Brca2* mRNA are expressed in all three germ layers and in virtually all cell types, with particularly high levels of expression noted in rapidly proliferating cell types undergoing differentiation such as the ventricular layer of the brain and germinal neuroblastic epithelium of the developing eye (10,19,27,29). In fact, spatial patterns of proliferation both during embryonic develoment and in adult tissues appear to be the strongest determinant of *Brca1* and *Brca2* mRNA expression patterns (19,29). Surprisingly, *in situ* hybridization analysis of serial sections of E18.5 embryos demonstrated essentially identical spatial patterns of expression of *Brca1* and *Brca2*, with high levels of expression observed in cellular compartments containing rapidly proliferating cells involved in differentiation, particularly developing neuroepithelium, toothbud, salivary gland, thymus, liver, lung, bowel and brown adipose tissue (Fig. 2) (19). *In situ* hybridization of E14–16 mouse embryos

Bright field
Brca2
Brca1
p21
Cephalic
Thoracic
Abdominal
mv
fv
po
oe
re
hf
lv
to
sg
tb
th
sc
bf
ht
di
li
lu
sk
bo
bl

demonstrated similar overlapping areas of *Brca1* and *Brca2* expression (19,20,28). The spatial coexpression of these genes contrasts markedly with that of the cell cycle regulatory protein $p21^{WAF1/CIP1}$ (Fig. 2). In addition to these initial studies demonstrating the striking spatial and temporal coexpression of *Brca1* and *Brca2* during embryonic development, *Rad51*, a protein that has been shown to interact directly or indirectly with both BRCA1 and BRCA2, is also spatially coexpressed with *Brca2* in the developing neuroepithelium of E8.5–E11.5 embryos (28,29,31).

## EXPRESSION IN ADULT TISSUES

*BRCA1* and *BRCA2* mRNA expression levels in adult tissues and cell lines are relatively low as assessed by *in situ* hybridization, Northern hybridization and RNase protection analysis (19,27). These assessments are supported by quantitative RNase protection and quantitative PCR analysis of *BRCA1* mRNA levels in cell lines, indicating that *BRCA1* mRNA is present in the HBL100 cell line at approximately 50 copies/cell, and at significantly lower levels in most other cell lines examined (21,32). This finding has important consequences for interpreting the physiological significance of experiments in which *BRCA1* expression is driven by relatively strong constitutive promoters since the resulting levels of *BRCA1* expression are almost undoubtedly supraphysiologic.

Similar to the pattern of expression in embryonic tissues, *BRCA1* mRNA can be detected in virtually all cell types and correlates most closely with proliferation (27,33). As originally reported, human *BRCA1* mRNA is expressed at highest levels in thymus and testis, with lower levels in breast and ovary (2). Similarly, the highest levels of *Brca1* mRNA expression in the FVB mouse are found in the testis, thymus and spleen with lower levels in breast, ovary and other tissues (2,10,13,27). Rat *Brca1* is also expressed at highest levels in the testis (34). *BRCA2* mRNA expression, like *BRCA1*, is found at highest levels in testis and thymus (14,19). Mouse *Brca2* mRNA is also expressed at highest levels in testis and thymus, with lower levels of expression in spleen, ovary, uterus, breast, and small intestine (17–19). Similarly, rat *Brca2* mRNA expression has been demonstrated at high levels in testis, with lower levels in thymus, spleen, mammary gland, ovary, prostate and heart. Thus, the tissue-specific patterns of *BRCA1* and *BRCA2* expression in adult tissues are remarkably similar in both human and mouse (14,19,20).

*In situ* hybridization analysis of adult tissues reveals that, as in the embryo, *Brca1* and *Brca2* are expressed in a wide variety of tissues and at highest levels in proliferating cellular compartments involved in differentiation, such as granulosa and thecal cells of developing ovarian follicles, endometrial glands in the uterus, the outer rim of the thymic cortex, and the basal epithelial cell layer in intestinal crypts of the small intestine and gastric glands in the stomach (19,27,29,35). The observation that *Brca1* and *Brca2* mRNA expression levels in the adult murine brain are low compared to other tissues is consistent with the positive correlation between *Brca1* and *Brca2* mRNA expression and cellular proliferation (10,18,19,27,36). In mouse testis, the highest levels of *Brca1* mRNA expression were detected in germ cells, specifically in pachytene spermatocytes and postmeiotic round spermatids, consistent with its suggested role in DNA repair and/or recombination (36). In other studies expression has been detected in both mitotic spermatogonia in addition to meiotic spermatocytes (29). Two groups have reported differences in the temporal patterns of *Brca1* mRNA expression relative to that of *Brca2* during spermatogenesis, though there is disagreement regarding the exact nature of these differences (19,29).

In light of the fact that much has been made of the relatively low level of evolutionary conservation between mouse and human BRCA proteins, it is interesting to note that the spatial pattern of *BRCA1* expression in primates is essentially identical to that described in the mouse (33). Specifically, *BRCA1* expression in

**Fig. 2.** *(opposite) In situ* hybridization analysis of *Brca1* and *Brca2* expression at E18.5. Bright field and dark field photomicrographs of serial frozen sections from the cephalic, thoracic or abdominal regions of day 18.5 mouse embryos hybridized to $^{35}$S-labeled antisense or sense probes for *Brca2*, *Brca1*, or $p21^{WAF1/CIP1}$. A pair of arrows shows the anatomical limits of a crosssection through a loop of bowel, and illustrates the difference in hybridization pattern between *Brca2*, *Brca1*, and $p21^{WAF1/CIP1}$ in the bowel. **Key:** brown adipose tissue (bf); urinary bladder (bl); bowel (bo); diaphragm (di); fourth ventricle (fv); hair follicle (hf); heart (ht); liver (li); lung (lu); lateral ventricle of brain (lv); mesencephalic vesicle (mv); roof of midbrain (md); midgut (mg); olfactory epithelium (oe); pons (po); respiratory epithelium (re); spinal cord (sc); submandibular gland (sg); skin (sk); stomach (st); tooth bud (tb); thymus (th); tongue (to); ventricular layer of brain (vl).

cynomolgus monkeys is high in granulosa cells in maturing follicles of the ovary, in glandular and luminal epithelial cells of the endometrium, in the crypts of the intestine and gastric glands of the stomach, and in seminiferous tubules of the testis in primary spermatocytes and spermatids (33).

Finally, as observed during fetal development, *in situ* hybridization analysis of *Brca1* and *Brca2* mRNA expression in tissues of the adult mouse has revealed essentially identical patterns of expression in multiple tissues including breast, ovary, duodenum, uterus and thymus (19). Subtle differences in spatial expression have been noted in some tissues (19). Interestingly, these expression patterns are similar to those described for the mouse *Rad51* gene in adult tissues, although as yet the comparison has not been performed directly (37).

## EXPRESSION IN THE MAMMARY GLAND

The spatial and temporal pattern of *Brca1* and *Brca2* expression in the mammary gland closely parallels that predicted from the patterns of expression observed in other tissues. Thus, in the breast as in other tissues both *Brca1* and *Brca2* are expressed in cellular compartments containing rapidly proliferating cells involved in differentiation, and both genes are expressed in a strikingly similar spatial and temporal pattern (10,19,27,29).

*Brca1* and *Brca2* are predominantly expressed in the mammary epithelium throughout the postnatal development of the gland, with lower but clearly detectable levels in the stroma, particularly that immediately adjacent to the epithelial compartment (10,27). This pattern is also seen in the mammary glands of cynomolgus monkeys (33). Both *Brca1* and *Brca2* are expressed at significantly higher levels in the female breast compared to the male breast, presumably reflecting the greater amount of mammary epithelium present in the adult female. RNase protection analysis has demonstrated that *Brca1* and *Brca2* mRNA levels in the mammary glands of 2 week and 5 week-old immature virgin female mice are significantly higher than those found in the mammary glands of 10 week and 15 week-old mature virgin females (19,27). Consistent with this finding *in situ* hybridization performed on the mammary glands of immature mice revealed higher levels of *Brca1* and *Brca2* mRNA in terminal end buds than in adjacent ducts or in ducts from sexually mature animals (19,27,29). The potential physiological significance of the elevation in *Brca1* and *Brca2* mRNA levels at age 2 weeks, prior to the onset of puberty and the formation of terminal end buds, is interesting to contemplate. *Brca1* and *Brca2* mRNA levels are both markedly upregulated early in pregnancy, a period during which alveolar buds begin the process of rapid proliferation and differentiation to form mature, milk-producing alveoli (10,19,27,29). *In situ* hybridization demonstrates that this upregulation of *Brca1* and *Brca2* expression occurrs preferentially in developing alveoli as compared with adjacent epithelial ducts, consistent with patterns of proliferation (19,21). *Brca1* and *Brca2* expression levels decline late in pregnancy, reaching their nadir during lactation and early postlactational regression, though expression levels remain above background even during these stages of development (10,19,27,29). Interestingly, the mammary glands of parous mice that have undergone four weeks of postlactational regression have consistently been shown to express higher levels of *Brca1* and *Brca2* mRNA than the mammary glands of age-matched virgin controls (19). Whether this observation reflects changes in the differentiated state of the mammary epithelium, changes in the distribution of mammary cell types in various phases of the cell cycle, or other as yet unrecognized factors remains to be elucidated.

Despite these similarities in *Brca1* and *Brca2* expression patterns, discrete differences have been noted in the quantitative patterns of expression of these two genes during mammary gland development. First, the magnitude of the up-regulation in *Brca1* mRNA levels that occurs either during early pregnancy, or in ovariectomized animals treated with estradiol and progesterone to simulate early pregnancy, was significantly greater than that observed for *Brca2* (19). When coupled with the observation that the ratio of mRNA expression in the mammary glands of adult females relative to adult males was significantly greater for *Brca1* than for *Brca2*, these observations raise the possibility that pathways activated by androgens and/or ovarian hormones may have differential effects on the regulation of *Brca1* and *Brca2* expression.

Regarding potential relationships between normal mammary gland development and reproductive endocrine risk factors for breast cancer, the observation that *Brca1* and *Brca2* are each up-regulated in the breast during puberty and pregnancy, periods of development associated with both increases in cellular proliferation and increases in breast cancer risk in humans, raises the possibility that the induction of *Brca1* and *Brca2*

expression is a protective response to proliferation (27). The observation that *Rad51* is similarly up-regulated in proliferating cells is consistent with this hypothesis, and suggests that induction of these genes may be a consequence of the need to activate DNA damage repair pathways to respond to the genomic damage that accompanies rapid proliferative states. Finally, since few somatic mutations in *BRCA1* or *BRCA2* have been identified in sporadic breast cancers, the intriguing possibility remains that the function of these cancer susceptibility genes in the mammary gland may be restricted to specific developmental stages.

## CELL CYCLE REGULATION OF EXPRESSION

The correlation between the proliferative status of tissues and *BRCA1* and *BRCA2* expression is also observed in cultured cells providing an opportunity for studies of the cell cycle-dependent expression of these genes (3,23,38–40). As in other contexts, the temporal pattern of *BRCA1* expression during the cell cycle is essentially indistinguishable from that of *BRCA2* (38–40).

*BRCA1* and *BRCA2* mRNA expression are tightly regulated during mammary epithelial proliferation. *BRCA1* and *BRCA2* mRNA levels are high in exponentially growing cells and decrease in cells made quiescent by confluence or growth factor withdrawal (3, 38–40). Cells arrested in G0 or early G1 express low levels of *BRCA1* and *BRCA2* mRNA. After release from serum starvation and reentry into the cell cycle, *BRCA1* and *BRCA2* mRNA levels progressively increase during G1 reaching maxima at the G1/S transition (3,38,39). Peak expression of *BRCA1* and *BRCA2* occurs just prior to expression of histones *H2A* and *H2B*, and parallels expression of the S-phase-dependent marker, cyclin A (23,38,39). Interestingly, these expression patterns are similar to that described for the mouse *Rad51* gene (37). Synchronization of cells at the G1/S boundary further demonstrates that induction of *BRCA1* and *BRCA2* mRNA expression occurs prior to, and independent of, the onset of DNA synthesis (38,40). The cell cycle dependence of expression has been documented by synchronization via starvation or treatment with reversible cell cycle inhibitors, and by centrifugal elutriation (36). Importantly, changes in BRCA1 and BRCA2 protein levels parallel changes in *BRCA1* and *BRCA2* mRNA levels. Specifically, as assessed in synchronized cells, the amount of BRCA1 and BRCA2 protein increases during progression through G1, peaking during S phase and remaining elevated in G2/M as compared with cells in G0/G1 (5,7,16,41). This finding suggests that at least in this context steady-state mRNA levels are the major determinants of protein levels (5,7,16,37,41,42).

The cell cycle-dependent pattern of expression of *BRCA1* and *BRCA2* has been demonstrated in human primary mammary epithelial cells derived from reduction mammoplasties, in multiple immortalized, nontumorigenic mouse and human mammary epithelial cell lines, and in a variety of breast cancer-derived cell lines as well as in other cancer-derived cell lines (3,37,39). In contrast, it has been reported that MCF10A cells do not exhibit the cell cycle-dependent, and growth factor-dependent changes in BRCA1 protein expression seen in other cell lines (8). Whether these differences are due to characteristics of the MCF10A cell line used or the experimental conditions employed is unclear. Nevertheless, in aggregate these results clearly demonstrate that proliferative stimuli modulate the mRNA expression of *BRCA1* and *BRCA2*.

## CELL CYCLE AND DNA DAMAGE-DEPENDENT REGULATION OF PHOSPHORYLATION

A key observation in studies of BRCA1 function was the observation that the mobility of BRCA1 in SDS polyacrylamide gels changes in a cell cycle-dependent manner, and that these changes in mobility reflect changes in the phosphorylation state of this protein (5,41,42). Changes in BRCA1 phosphorylation parallel cell cycle-dependent changes in protein level (7,41). Alterations in BRCA1 mobility in SDS polyacrylamide gels observed during the cell cycle are abolished by treatment of immunoprecipitates with phosphatase (5,7,41,42). Phosphatase treatment of BRCA1 isolated from G0/G1 enriched cells results in an increased electrophoretic mobility, suggesting that BRCA1 is partially phosphorylated even during G0/G1 (7). BRCA1 undergoes hyperphosphorylation during late G1 and S phases of the cell cycle and is transiently dephosphorylated early after M phase as assessed by imposing a reversible cell cycle block (7,41,42). Similar results are seen in untreated cells separated by centrifugal elutriation (7). The kinase(s)

responsible for the cell cycle dependent changes in BRCA1 phosphorylation is presently unknown.

BRCA1 has subsequently been shown to be a serine phosphoprotein (5,7). Two-dimensional tryptic peptide analysis of BRCA1 isolated from HeLa cells and from BRCA1 overexpressing 293T cells has revealed that BRCA1 is phosphorylated predominantly on serine and weakly on threonine (7). A low degree of phosphorylation has been detected on tyrosine by one group using phosphoamino acid analysis (6). Conflicting results have been obtained by other groups by immunoblotting with anti-phosphotyrosine antibodies (42,43).

In addition to cell cycle-dependent changes in BRCA1 phosphorylation, treatment of S-phase cells with a variety of DNA damaging agents, including ultraviolet radiation (UV), hydroxyurea, gamma radiation, mitomycin C, and hydrogen peroxide leads to phosphorylation of BRCA1 within approximately 20 minutes (41,42). Phosphorylation of BRCA1 in G1 phase cells does not occur following exposure to hydroxyurea, mitomycin C or low-dose UV-treated cells, although higher levels of UV irradiation are able to shift the G1 form of BRCA1 (41). Together these observations strongly suggest a role for BRCA1 in sensing or responding to DNA damage. This hypothesis is further supported by the physical association of both BRCA1 and BRCA2 with Rad51. In aggregate, current evidence favors the existence of a large multiprotein complex in the nucleus involved in the of sensing and/or response to DNA damage (1).

## HORMONAL REGULATION OF EXPRESSION

It is likely that the effects of hormones on *BRCA1* and *BRCA2* expression can be best understood as a consequence of the cell cycle-dependence of *BRCA1* and *BRCA2* expression. Initial studies of the *in vivo* pattern of *Brca1* gene expression were interpreted as reflecting patterns or proliferation and differentiation rather than direct consequences of hormone exposure (10,21). For instance, the upregulation of *Brca1* and *Brca2* expression that occurs in the mammary gland in response to either pregnancy or treatment with estradiol and progesterone could mean either that the expression of these genes is directly regulated by steroid hormones, or that the induction of *Brca1* and *Brca2* expression is an indirect consequence of the rapid proliferation and differentiation of the mammary epithelium that occurs in response to these hormones. The observation that *Brca1* expression is markedly elevated in virtually all rapidly proliferating cellular populations regardless of whether they are hormonally-regulated was taken as support for the hypothesis that *Brca1* mRNA expression in the mammary gland reflects cellular proliferation induced by hormonal stimulation, rather than a direct effect of these hormones on gene expression *per se* (27).

Subsequent *in vivo* and *in vitro* studies have borne out this hypothesis for both *Brca1* and *Brca2*. In particular, *Brca1* and *Brca2* have been shown to be expressed at highest levels in granulosa and thecal cells of the small and medium ovarian follicles that grow independently of hormonal stimulation (29,35). Impressively, *Brca1* and *Brca2* continue to be expressed at high levels in this class of follicles even in the absence of hormonal stimulation, as shown by studies in both hypophysectiomized and estrogen-receptor-deficient mice (29,35). Consistent with this idea, *Brca1* and *Brca2* expression uniformly correlated with S-phase proliferating cell nuclear antigen expression (29,35).

Similarly, *in vitro* studies have demonstrated that treatment of estrogen receptor-positive mammary epithelial cell lines with estradiol stimulates proliferation of these cell lines and results in a coordinate increase in *BRCA1* and *BRCA2* mRNA levels (23,44,45). These estradiol-induced increases in *BRCA1* and *BRCA2* expression occur in parallel with the increases in S-phase-dependent markers such as cyclin A, rather than in parallel with classical estrogen-responsive genes such as pS2 (23). In addition, other hormones and growth factors that stimulate mitogenesis have a similar effect, including insulin like growth factor-1, epidermal growth factor, and progesterone (23,45). Conversely, estrogen depletion results in a reduction in *BRCA1* mRNA and protein levels in estrogen receptor-positive cell lines, as does treatment with agents such as TGF-β that inhibit mammary epithelial proliferation (3). Moreover, induction of *BRCA1*, but not pS2, is blocked by cycloheximide indicating that the estradiol-induced upregulation of *BRCA1* and *BRCA2* expression requires *de novo* protein synthesis and is therefore indirect (44,45). These observations strongly suggest that the ability of hormones to regulate *BRCA1* and *BRCA2* gene expression in specific cell types is primarily due to the ability of these agents to modulate proliferation in these cells, rather than to specific and direct effects of hormones on *BRCA1* and *BRCA2* expression *per se*.

## REGULATION OF *BRCA1* AND *BRCA2* EXPRESSION IN RESPONSE TO OTHER STIMULI

Despite the strong correlation between *Brca1* and *Brca2* expression and proliferative status, an increasing number of instances have been described in which the expression of these genes appears to be influenced by factors other than proliferation. For instance, analysis of *Brca1* and *Brca2* mRNA levels in HC11 mammary epithelial cells reveals coordinate up-regulation in postconfluent cells treated with insulin and glucocorticoids (39). Steady-state levels of *Brca1* and *Brca2* mRNA expression in this context increase progressively over the course of several days to levels as high as those found in actively proliferating cells, despite the fact that rates of proliferation in these cells remain low and relatively constant. This up-regulation requires a serum factor that is removed by charcoal stripping (39).

Similarly, other investigators have argued that the dependence of *Brca1* and *Brca2* expression on proliferation is also abrogated in selected other tissues, such as in the testis and in differentiated neurons in the brain (29). Interestingly, *BRCA1* mRNA and protein expression have been reported to be transiently elevated in SKOV-3 cells following treatment with cis-diamminedichloroplatinum(II) (CDDP), stably elevated in A2780 cells chronically exposed to adriamycin or cisplatin, and stably elevated in CDDP-resistant variants of MCF-7 cells and SKOV-3 ovarian carcinoma cells (42,46). Confirmation that these increases in *BRCA1* expression are genuinely independent of proliferation will ultimately require elucidation of the pathways responsible for these effects. Nevertheless, studies of other tumor suppressor genes would suggest that *BRCA1* and *BRCA2* expression are likely to be controlled in a complex manner and to respond to diverse stimuli.

## EXPRESSION IN BREAST CANCER

As expected on the basis of the widespread distribution of *BRCA1* expression *in vivo*, this gene is expressed in a wide variety of tumor cell types. It has been reported that in sporadic breast cancer *BRCA1* mRNA levels decrease during the transition from carcinoma *in situ* to invasive cancer (47). Relative to normal mammary epithelium, *BRCA1* mRNA expression was found to be increased in ductal carcinoma *in situ* and decreased in invasive cancer. Of four informative sporadic breast cancer cases examined, decreased *BRCA1* expression appeared to involve both *BRCA1* alleles in three cases (47). Whether the fourth case represented down-regulation of one allele by mutation, or loss of heterozygosity was not reported (41). If confirmed, it is unclear whether this downregulation of *BRCA1* expression in invasive cancer is more likely to represent selection for decreased BRCA1 activity in the course of tumor progression (i.e. a causal event) or a secondary effect occurring as a consequence of changes in upstream regulatory pathways controlling *BRCA1* expression. In this regard, it is important to note that the possibility of confounding effects of differences in proliferation rates on *BRCA1* expression were not assessed in this study, as it was performed prior to the recognition of the importance of this variable.

It has been somewhat difficult to reconcile the observed decrease in *BRCA1* expression in invasive breast cancers with observations that levels of *Brca1* mRNA expression in mammary epithelial cells *in vitro* are similar in transformed and non transformed cells (10,27,39). Furthermore, *Brca1* mRNA expression levels have been shown to be similar in normal rat mammary glands compared with mammary tumors induced either by the carcinogens DMBA or NMU, or by the activated-*neu* or activated-*ras* oncogenes (34). Finally, quantitative mRNA *in situ* hybridization performed on archival tumor specimens from patients with characterized *BRCA1* mutations and from patients with sporadic breast cancers revealed that, while *BRCA1* mRNA levels were invariably low in tumors from *BRCA1* mutations carriers relative to surrounding normal epithelium, only one third of sporadic tumors showed decreased *BRCA1* mRNA levels relative to surrounding normal breast epithelium (48). Clearly, additional data are needed to fully understand these findings.

The observation that somatic mutations in the coding regions of *BRCA1* and *BRCA2* are extremely rare in sporadic tumors, as well as the observation that *BRCA1* mRNA expression may be reduced in invasive breast tumors, has prompted a search for either regulatory mutations in the promoter regions of these genes or epigenetic mechanisms such as methylation that might account for their loss of activity in tumors in the absence of somatic mutations. The presence of regulatory mutations in familial breast cancer families was previously inferred from the existence of individuals heterozygous for a series of polymorphisms in the *BRCA1* gene, while mRNA isolated from these indi-

viduals appeared homozygous at these loci (2). Such inherited regulatory mutations could potentially cause reduced transcription, decreased mRNA stability, or aberrant splicing of the *BRCA1* transcript (2). Similar loss of transcription from one *BRCA1* allele has been reported in other families (49,50). Interestingly, the mutation in at least one of these families has been identified as a 14 kb deletion that removes exons 1a, 1b and 2 of *BRCA1*, thereby deleting both transcriptional initiation sites (49). Unequal crossover between Alu repeats appeared to be the most likely cause for this deletion, a significant observation given the fact that the *BRCA1* genomic sequence contains many such repeats. It has been proposed that 10–30% of germline mutations in *BRCA1* may be large deletions, and that up to half of these may affect the promoter region (51).

The observation that methylation of tumor suppressor genes such as *MTS1*, *RB1* and *VHL* has been implicated in decreased expression of these genes in cancer, suggests that epigenetic events may also play a role in sporadic breast cancer. Both the *BRCA1* and *BRCA2* genes are preceded by CpG islands (52). In contrast to most of the genome in which CpG dinucleotides are underrepresented and are constitutively methylated, CpG islands are typically unmethylated and represent potential sites for gene regulation via DNA methylation. The *BRCA1* CpG island extends over 1200 bp and includes exons 1a and 1b of *BRCA1* as well as its associated promoter region (53). In one study, the *BRCA1* promoter region in two of seven sporadic breast carcinomas was found to be hypermethylated (53). Hypermethylation was not detected in normal breast tissue samples or in samples of peripheral blood mononuclear cells. In a related study, an independent group identified CpG methylation in two of six breast carcinomas and two of five ovarian carcinomas, but not in normal tissues (54). The significance of these observations for the process of carcinogenesis is unknown. At present, compelling evidence for methylation in the *BRCA2* promoter region in breast or ovarian cancer cell lines is lacking (55).

## PROMOTER ANALYSIS

*BRCA1* transcription initiates from either of two sites separated by 277 bp and is under the control of two promoters that generate two distinct transcripts, alpha and beta, containing the alternatively spliced first exons, 1A and 1B (Fig. 3) (52,56,57). While both transcripts are expressed in most tissues and cell lines that have been examined, the alpha promoter appears to be the more potent of the two (54). Both the *BRCA1* alpha and beta promoters are TATA-less, although each contains GC-rich regions and has motifs similar to initiator elements as well as putative binding sites for the transcription factor, Sp1 (52,56,58). A role for a CCAAT box located in the intergenic region has been suggested based on transient transfection experiments (57). A variety of putative binding sites for other known transcription factors have been identified based on computer analysis although few have been directly tested. Neither promoter contains a classical estrogen response element. However, modest 1.5–2-fold increases in transcriptional activity in response to estradiol have been demonstrated in MCF-7 cells for fragments of the alpha and beta promoters containing either an AP1 site or an alternative estrogen response element (57). The failure of other investigators to detect a response to estrogen stimulation using genomic fragments near the 5′ end of the *BRCA1* gene may be due to differences in the genomic regions contained within these respective constructs (45).

In the human, a 30 kb tandem duplication of the genomic region containing the 5′ end of the *BRCA1* gene and a gene referred to as *NBR2* has been delineated (59) Fig. 3. The 5′ end of *NBR2* was initially believed to be a pseudocopy of *NBR1*, originally referred to as *1A1–3B*, which is itself located in a head-to-head configuration adjacent to a pseudocopy of the 5′ end of *BRCA1* that encompasses copies of exons 1a, 1b and 2 (59). Thus *BRCA1* is located in a head-to-head configuration with *NBR2*, while *NBR1* lies in a head-to-head configuration with a pseudocopy of the 5′ end of *BRCA1* (52,59,60). The transcription start sites of *BRCA1* and the adjacently located gene, *NBR2*, are only 218 bp apart. Promoter alpha is shared with the adjacent *NBR2* gene and is bi-directional (57). This complex arrangement raises the possibility that homology-mediated genetic rearrangements may lead to regulatory mutations in this gene that might not be detected by methods typically employed to search for *BRCA1* mutations (60).

The genomic organization of the 5′-flanking region of mouse *Brca1* gene is markedly different from that found in humans (61). The 30 kb genomic DNA fragment found in humans containing the *NRB2* gene and a pseudocopy of the 5′ end of the *BRCA1* gene is absent in mice (59). Moreover, alternative exon 1B of the human *BRCA1* transcript is not found in the mouse (57). These observations suggest that the mouse *Brca1* gene is unlikely to be regulated by two promot-

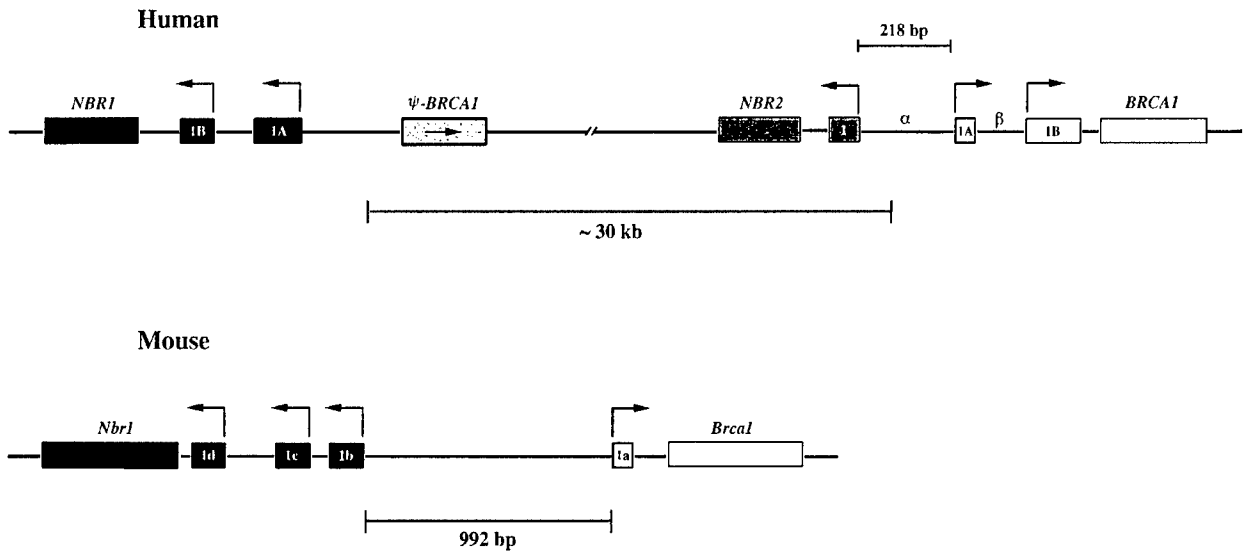


**Fig. 3.** Genomic structure of *BRCA1* locus and promoter region. Key: Open boxes, *BRCA1*; solid boxes, *NBR1*, light-shaded box, *BRCA1* pseudogene (ψ-*BRCA1*); dark-shaded box, *NBR2*. Alternate first exons of *BRCA1* and *NBR1* genes are shown. Rectangular arrows indicate transcription initiation sites and the direction of transcription. An internal arrow denotes the orientation of the BRCA1 pseudogene. The two promoter regions of human *BRCA1* are indicated as α and β. The diagram is not drawn to scale. Distances between the transcriptional initiation sites of *BRCA1* and *NBR2*, and between those of *Brca1* and *Nbr1* are indicated, as is the 30 kb duplicated genomic region of the human locus. Modified based on refs 56–61.

ers (57). While it has been suggested that the marked differences in the promoter regions of mouse and human *BRCA1* imply that the temporal and spatial pattern of expression of this gene may differ between the two species, it is clear that the temporal pattern of *BRCA1* expression during the cell cycle is indistinguishable for the mouse and human gene, and that the spatial pattern of *BRCA1* expression in multiple adult tissues is strikingly similar in mice and primates.

## CONCLUSIONS AND FUTURE DIRECTIONS

Studies of the expression pattern of *BRCA1* and *BRCA2* have revealed multiple clues regarding the likely functions of these proteins. The best index of expression for these genes in a given population of cells is their proliferative status. As a result, any studies comparing expression levels of *BRCA1* or *BRCA2* between different tissues, cellular populations or developmental states must control for effects of proliferation on expression levels. Similarly, as a result of the finding that *BRCA1* and *BRCA2* expression levels are relatively low, caution must be used in interpreting studies in which these genes are massively overexpressed.

Notably, despite the relatively low extent of evolutionary conservation of the coding sequence and promoter regions of these genes, the patterns of regulated expression observed in the mouse, rat, monkey and human are nearly identical. These findings strongly argue that the evolutionary functions of these genes have been highly conserved. It will be of particular interest to determine the elements in the *BRCA1* and *BRCA2* promoters responsible for their cell cycle-dependent patterns of expression, and to compare the manner in which this regulation is achieved both between these different genes and for the same gene among different species. While it currently appears that the effects of hormones on *BRCA1* and *BRCA2* expression are primarily a consequence of their effects on proliferation and differentiation, further promoter analysis may be required to resolve this issue definitively.

One of the most striking findings to emerge from these studies is that *Brca1* and *Brca2* are expressed in similar tissue-specific patterns, at similar levels in a given tissue, and in similar cellular compartments within each tissue. In fact, during fetal development and in multiple adult tissues, the spatial and temporal patterns of *Brca1* and *Brca2* expression are virtually indistinguishable. This similarity is particularly evi-

dent during postnatal mammary gland development in the mouse, as each of these genes is up-regulated during puberty and pregnancy. A potential basis for this similarity is provided by the observation that *Brca1* and *Brca2* expression are coordinately regulated in proliferating and differentiating mammary epithelial cells *in vitro*. The remarkable extent to which *Brca1* and *Brca2* expression are coordinately regulated indicates that these genes are induced by similar stimuli, and suggests that they may function in overlapping pathways. Interestingly, similar overlapping expression patterns may be seen among subunits of heteromeric proteins. Whether or not the BRCA1 and BRCA2 proteins physically interact is currently a topic of intense investigation.

In contrast to recent advances in understanding *Brca1* and *Brca2* function in embryonic cells, relatively little is known about the function of these molecules in mammary epithelial cells. This deficiency is particularly relevant given that breast cancer is the predominant phenotype associated with mutation of these genes. For instance, the fact that germline *BRCA1* and *BRCA2* mutations specifically predispose carriers to breast cancer may relate to mammary-specific functions of these molecules. Moreover, there is as yet no explanation for the observation that *BRCA1* and *BRCA2* mutations appear to cause breast cancer only when present in the germline, since somatic mutations in these genes are not found in sporadic breast cancers. This finding may indicate that these molecules function in specific stages of mammary gland development, as might be predicted from their tightly regulated expression during mammary gland development. It should also be noted that *Brca1* and *Brca2* may function differently in embryonic versus adult cells. These findings, when considered with the possiblity that *BRCA1* and *BRCA2* may have mammary epithelial-specific functions, strongly argue that a full understanding of the role played by these genes in breast cancer susceptibility will require that their functions be studied directly in the mammary epithelium.

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# Mammary Gland Development, Reproductive History, and Breast Cancer Risk[1]

**Lewis A. Chodosh,[2] Celina M. D'Cruz, Heather Perry Gardner, Seung I. Ha, Sandra T. Marquis, Jayant V. Rajan, Douglas B. Stairs, James Y. Wang, and Man Wang**

*Departments of Molecular and Cellular Engineering [L. A. C., C. M. D., H. P. G., S. I. H., S. T. M., J. V. R., D. B. S., J. Y. W., M. W.], and Department of Medicine, Division of Endocrinology, Diabetes and Metabolism [L. A. C.], Stellar-Chance Laboratories, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6100*

## Abstract

**The observation that normal pathways of differentiation and development are invariably altered during the process of carcinogenesis implies an intrinsic relationship between these processes. This relationship is particularly evident in the breast, as exemplified by the existence of endocrine risk factors for breast cancer that are related to the timing of normal developmental events. Understanding the mechanisms by which normal developmental events alter breast cancer risk is a central focus of our laboratory. Herein, we describe three approaches being taken in our laboratory toward defining the molecular basis of this relationship. These include: determining the roles played by the tumor suppressor genes, *BRCA1* and *BRCA2,* in the normal differentiation and development of the breast; studying the function of three novel protein kinases identified in our laboratory in mammary epithelial development; and defining the molecular and cellular changes that occur in the breast as a result of reproductive events known to influence breast cancer risk.**

## Introduction

A basic tenet emerging from studies in cancer biology is that normal pathways of differentiation and development are inevitably disrupted during the process of carcinogenesis. This implies an intrinsic relationship between these processes. The existence of endocrine risk factors for breast cancer that are related to the timing of normal developmental events such as menarche, menopause, and age at first full-term pregnancy epitomizes this relationship. The recognition that breast cancer risk is determined in part by the same reproductive endocrine events that drive mammary gland development argues that mammary gland development and mammary carcinogenesis are fundamentally related.

One of the most intriguing examples of this principle is the observation that women who undergo their first full-term pregnancy early in life (*i.e.*, early parity) have a significantly reduced lifetime risk of breast cancer (1). The magnitude of this parity-induced protection against breast cancer is similar in many countries and ethnic groups, regardless of endemic incidence. This suggests that protection results from an intrinsic effect of parity on the biology of the breast rather than from extrinsic factors specific to a particular environmental, genetic, or socioeconomic setting. This conclusion is bolstered by the observation that rats that have previously undergone a full-term pregnancy are resistant to the induction of breast cancer by administration of the carcinogen DMBA,[3] as compared to age-matched nulliparous controls (2, 3). Therefore, both human epidemiology and animal model systems support the conclusion that an early first full-term pregnancy results in a permanent change in the breast, either directly or indirectly, that confers a decreased risk for the subsequent development of breast cancer. Although this effect has been hypothesized to result from the impact of terminal differentiation on the susceptibility of the mammary epithelium to carcinogenesis, the molecular and cellular basis for this phenomenon is unknown.

A second illustration of this principle comes from the observation that breast cancer risk attributable to exposure to ionizing radiation is a function of age at the time of exposure. Specifically, studies of women who received mantle irradiation for Hodgkin's disease or who underwent repeated fluoroscopy in the course of treatment for tuberculosis have demonstrated that breast cancer risk is significantly greater in women who were exposed to ionizing radiation during adolescence as compared to women exposed at later ages (4, 5). Analogously, nulliparous rats fed DMBA are more likely to develop breast cancer if they are exposed during puberty rather than as mature adults (6). Interestingly, epidemiological studies suggest that the increased susceptibility of the immature human breast to early events in carcinogenesis may occur prior to as well as during puberty. Studies of survivors from Hiroshima and Nagasaki indicate that the greatest increase in breast cancer risk occurred in women who were less than 10 years old at the time of exposure (7). The observed increase in breast cancer incidence in women irradiated during the first year of life for presumed thymic enlargement is perhaps an even more impressive illustration of this principle, given the rudimentary state of the mammary gland at this age (8). Together, these studies suggest that the susceptibility of the mammary gland to carcinogenesis is related to the gland's developmental state at the time of exposure to mutagenic agents and that the immature breast is particularly susceptible to early events in carcinogenesis.

Understanding the molecular and cellular mechanisms by which normal developmental events alter breast cancer risk is a central goal of our laboratory. We believe that achieving this goal requires a more complete understanding of the manner in which hormones and reproductive history alter subpopulations of epithelial cell types present in the breast and of the roles played by key regulatory molecules in these processes. Toward this end, we are currently focusing on: (*a*) determining the roles played by the tumor suppressor genes, *BRCA1* and *BRCA2,* in the normal differentiation and development of the breast; (*b*) studying the function of three novel protein kinases identified in our laboratory in mammary epithelial development and carcinogenesis; and (*c*) defining the molecular and cellular changes that occur in the breast as a result of reproductive events known to influence breast cancer risk.

## Tumor Suppressor Genes: *BRCA1* and *BRCA2*

The epidemiological relationship between development and carcinogenesis is illustrated on a molecular and mechanistic level by the existence and function of tumor suppressor genes such as *p53*, the Wilms' tumor gene (*WT1*), and the retinoblastoma susceptibility gene (*RB*). Germ-line mutations in these genes are associated with inherited cancer predisposition syndromes (9). The cloning and analysis of several tumor suppressor genes has revealed that they frequently

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[2] To whom requests for reprints should be addressed, at Department of Molecular and Cellular Engineering, Stellar-Chance Laboratories, Room 309A, University of Pennsylvania School of Medicine, 422 Curie Boulevard, Philadelphia, PA 19104-6100. Phone: (215) 898-1321; Fax: (215) 573-8590; E-mail: chodosh@mail.med.upenn.edu.

[3] The abbreviations used are: DMBA, 7,12-dimethylbenz[*a*]anthracene; EGFR, epidermal growth factor receptor.

encode proteins that act as negative regulators of cell proliferation, exert cell cycle checkpoint control function, or maintain genome integrity (10, 11). In addition, the targeted deletion of these genes in mice frequently results not only in increased susceptibility to cancer but also in abnormalities in proliferation, apoptosis, differentiation, and development (10, 12). As such, one approach to elucidating the relationship between mammary gland development and carcinogenesis is to determine the function of tumor suppressor genes known to be involved in the pathogenesis of breast cancer.

Genetic analysis of families in which multiple individuals have developed breast cancer suggests that 5–10% of breast cancer cases result from the inheritance of germ-line mutations in autosomal dominant susceptibility genes (13, 14). Over the past 4 years, several of these breast cancer susceptibility genes have been isolated by positional cloning, including *BRCA1* and *BRCA2* (15–19). Tumors arising in patients with germ-line mutations in either *BRCA1* or *BRCA2* typically display loss of the corresponding wild-type allele, suggesting that *BRCA1* and *BRCA2* are tumor suppressor genes (20–22). Interestingly, *BRCA1* and *BRCA2* mutations have not been identified in sporadic breast cancers, despite the fact that 25–30% of sporadic breast cancers show loss of heterozygosity at these loci (16, 23–26). This raises the intriguing possibility that the normal functions of these genes are temporally and/or developmentally restricted.

Recently, important clues to BRCA1 and BRCA2 function have come from biochemical studies demonstrating that treatment of cells with a variety of DNA-damaging agents leads to the rapid phosphorylation of BRCA1 (27, 28). Moreover, both BRCA1 and BRCA2 have been shown to directly or indirectly bind to RAD51, a homologue of RecA that has been implicated in DNA repair and recombination (29–32). These and other observations have led to the hypothesis that BRCA1 and BRCA2 are involved in the cellular response to DNA damage. Consistent with this hypothesis, embryonic cells from mice homozygous for mutations in the *Brca2* locus have an increased sensitivity to DNA-damaging agents (30, 33, 34). It is interesting to speculate that the developmental regulation of *BRCA1* and *BRCA2* expression or function may contribute to the age-dependent susceptibility of the breast to ionizing radiation-induced carcinogenesis described above.

The markedly elevated risk of breast cancer observed in women carrying germ-line mutations in *BRCA1* and *BRCA2* strongly suggests that these gene are critical for the properly regulated growth of mammary epithelial cells. As a first step toward understanding the developmental role of *BRCA1* and *BRCA2*, we have analyzed the spatial and temporal expression of the murine homologues of these genes during embryogenesis, in the mammary gland during postnatal development, and in adult tissues (35, 36). These studies reveal that expression of both *Brca1* and *Brca2* are tightly regulated during mammary gland development. For example, *Brca1* and *Brca2* expression levels in the mammary glands of adolescent female mice undergoing ductal morphogenesis are significantly higher than those found in the mammary glands of mature females in whom ductal morphogenesis has been completed (35, 36). This temporal pattern of expression is explained in part by the observation that *Brca1* and *Brca2* are expressed at high levels in terminal end buds, which are puberty-specific structures that contain rapidly proliferating cells undergoing differentiation (35–37). *Brca1* and *Brca2* mRNA levels are also markedly up-regulated in the mammary gland early in pregnancy, a period during which alveolar buds begin the process of rapid proliferation and differentiation to form mature, milk-producing alveoli (35–38). This up-regulation of *Brca1* and *Brca2* expression occurs preferentially in developing alveoli as compared to adjacent epithelial ducts, consistent with patterns of proliferation (35, 36). Indeed, at virtually all stages of development, *Brca1* and *Brca2* expression are restricted to cellular compartments actively involved in proliferation and differentiation. These patterns of expression suggest that these tumor suppressor genes may play a role in the normal development of the breast and other tissues.

The spatial and temporal patterns of *Brca1* and *Brca2* expression during development likely reflect the fact that expression of these genes is tightly regulated as a function of proliferation. We have shown that *Brca1* and *Brca2* mRNA levels are high in exponentially growing cells and low in quiescent cells (39). During progression through the cell cycle, *Brca1* and *Brca2* mRNA levels increase during $G_1$ and attain maximal levels at the $G_1$-S transition (39). Similar observations have been made for human BRCA1 and BRCA2 at both the mRNA and protein levels (39–46). These findings clearly demonstrate that proliferative stimuli modulate the expression of these genes. Despite the strong correlation between *Brca1* and *Brca2* expression and proliferative status, the expression of these genes also appears to be influenced by factors other than proliferation. For example, we have shown that *Brca1* and *Brca2* mRNA levels are coordinately up-regulated in postconfluent HC11 mammary epithelial cells during differentiation as well as following treatment with insulin and glucocorticoids (39). *Brca1* and *Brca2* expression increase in this setting to levels as high as those found in actively proliferating cells, despite the fact that cellular proliferation rates remain low under these experimental conditions. Together, these observations imply that *Brca1* and *Brca2* may be involved in the processes of proliferation and differentiation in the breast.

A particularly intriguing finding of our studies has been the striking degree to which *Brca1* and *Brca2* are temporally and spatially coexpressed at the mRNA level (36). We have found that *Brca1* and *Brca2* are expressed at similar levels in a similar set of tissues and in similar cellular compartments within those tissues. In fact, the developmental expression patterns of these two putative tumor suppressor genes are essentially identical during embryogenesis and in multiple tissues of the adult. This similarity is particularly evident during postnatal mammary gland development as *Brca1* and *Brca2* expression are each up-regulated during puberty and pregnancy. The coordinate induction of these genes in proliferating and differentiating mammary epithelial cells *in vitro* may provide a cellular basis for this similarity (39). These findings suggest that similar pathways and stimuli regulate the expression of *Brca1* and *Brca2* in multiple cell types. Taken together with the fact that inherited mutations in either *BRCA1* or *BRCA2* predispose mammary epithelial cells to transformation, the striking similarities in *Brca1* and *Brca2* expression patterns formed the initial basis for speculation that these genes may function in overlapping pathways and may even directly interact.

As alluded to above, no somatic mutations have been identified in *BRCA1* or *BRCA2* in sporadic breast cancers. This puzzling observation could be explained if the function of these cancer susceptibility genes in the mammary gland were restricted to specific developmental stages, as might be suggested by the tightly regulated expression that these molecules exhibit during mammary gland development. Similarly, in light of the proposed relationship between normal mammary gland development and reproductive risk factors for breast cancer, it is interesting to note that *Brca1* and *Brca2* are each up-regulated in the breast during puberty and pregnancy because these stages of development are each associated with increases in cellular proliferation as well as increases in breast cancer risk. Potentially, the induction of *Brca1* and *Brca2* expression during these developmental stages may be a protective response to proliferation or to DNA damage that accompanies proliferation, as suggested by the observation that *Rad51* is also up-regulated in proliferating cells (35, 47).

Our laboratory has chosen to focus on understanding BRCA1 and BRCA2 function in mammary epithelial cells because considerably

less is known about their function in this context and because breast cancer is the most important clinical phenotype associated with germ-line mutations in these genes. Specifically, we are interested in those aspects of mammary gland biology responsible for the observation that women carrying germ-line mutations in *BRCA1* and *BRCA2* preferentially develop cancer of the breast. Because this may ultimately relate to mammary-specific functions of these molecules, a complete understanding of the role played by these genes in breast cancer susceptibility will almost certainly require that their functions be studied directly in the mammary epithelium. As such, we are analyzing the impact of altering *BRCA1* and *BRCA2* expression levels on proliferation, differentiation, and DNA repair in the mammary epithelium using *in vivo* and *in vitro* model systems. These studies may provide insight into mechanisms of growth control and DNA damage response in normal mammary epithelial cells as well as serve as a foundation for understanding how the absence or mutation of these molecules promotes carcinogenesis.

## Novel Protein Kinases

A second approach to investigating the relationship between development and carcinogenesis in the breast is to study members of a family of regulatory proteins that are typically involved in differentiation, development, and carcinogenesis. Analysis of these processes in a variety of model systems has underscored the key role frequently played by protein kinases. Many protein kinases function as intermediates in mitogenic signal transduction pathways or encode growth factor receptors whose overexpression, aberrant expression, or mutation to ligand-independent activated forms results in transformation. Several members of the protein kinase family have been shown to be involved in the development of breast cancer both in humans and in rodent model systems including the epidermal growth factor receptor, the insulin-like growth factor-I receptor, the fibroblast growth factor receptor family, HER2/Neu, Met, and Src. For instance, amplification and overexpression of *HER2/Neu* and *EGFR* have each been correlated with aggressive tumor phenotype and poor clinical prognosis. Similarly, overexpression of certain protein kinases or of their ligands in transgenic animals results in malignant transformation of the mammary epithelium. To date, however, evidence for a causal role of protein kinases in the initiation and progression of breast cancer exists for only a few members of this family of proteins. For this reason, we embarked on a screen designed to identify tyrosine kinases and serine-threonine kinases expressed in the murine breast during normal development and in breast cancer.

First-strand cDNA was prepared from mRNA isolated either from mammary glands of mice at specific developmental stages or from a series of mammary epithelial cell lines derived from breast tumors that arose in transgenic mice expressing either the activated *neu*, c-*myc*, H-*ras*, or *int2* oncogenes (48–50). Degenerate PCR was used to amplify kinase catalytic subdomains VI–IX, and the resulting cDNA clones were screened to identify those harboring catalytic domain fragments of protein kinases (51–53). This screen identified 41 kinases: 33 tyrosine kinases and 8 serine-threonine kinases, 3 of which are novel.[4] We have characterized the temporal and spatial expression of these kinases during mammary gland development as well as in a panel of mammary epithelial cell lines derived from breast tumors arising in transgenic mice expressing either the activated *neu*, c-*myc*, H-*ras*, or *int2* oncogenes.[4] This analysis has revealed that many of these kinases are preferentially expressed in the breast during specific stages of puberty, pregnancy, lactation, and postlactational regression.

Our laboratory has subsequently focused on the function of three novel serine-threonine kinases identified in our screen: *Hunk*, *Punc*, and *Krct*. The novel protein kinase, *Hunk*, was initially isolated from a mammary epithelial cell line derived from a breast tumor that arose in a transgenic mouse expressing the *neu* oncogene (54).[4,5] Analysis of sequence homology within a portion of the catalytic domain of *Hunk* suggests that it is a serine/threonine kinase with highest homology to the *SNF1* kinase family. The novel protein kinase, *Punc*, was initially isolated from the mammary glands of mice undergoing early postlactational regression.[4,6] The catalytic domain of *Punc* is 60% identical at the amino acid level to calcium/calmodulin-dependent protein kinase I and shares a lower homology with other members of the calcium/calmodulin-dependent kinase family (55).[6] *Krct* appears to represent a new family of mammalian protein kinases and is most closely related to a protein kinase recently identified by the yeast genome project that does not fall into any of the families of protein kinases previously identified in yeast (54).

*Hunk* and *Punc* appear to be particularly relevant to studies of the relationship between mammary gland development and carcinogenesis by virtue of their patterns of expression.[7,8] Specifically, *Hunk* is expressed at low levels in the mammary glands of immature and mature virgin animals and undergoes a dramatic up-regulation of expression during early pregnancy. *Hunk* expression rapidly drops to basal levels by midpregnancy and decreases further during lactation and early postlactational regression. Like *Hunk*, *Punc* expression is also up-regulated in the mammary epithelium during pregnancy. However, unlike *Hunk*, maximum levels of *Punc* expression occur late in pregnancy just prior to parturition.

To determine whether the developmental changes in *Hunk* and *Punc* expression observed during pregnancy represent global changes in expression occurring throughout the mammary gland or changes in the abundance of an expressing subpopulation of cells, we have defined the spatial pattern of expression of these kinases.[7,8] This was of particular interest because the expression of several protein kinases has been shown to be cell lineage restricted, thereby permitting their use as markers for biologically interesting subpopulations of cells. Examination of the spatial pattern of *Hunk* and *Punc* expression revealed that throughout the course of mammary development both kinases are expressed predominantly in the mammary epithelium. Interestingly, the expression of each of these kinases in the mammary epithelium is strikingly heterogeneous, with the greatest number of *Hunk*-expressing cells being observed at day 7 of pregnancy and the greatest number of *Punc*-expressing cells being observed at day 20 of pregnancy. This pattern of expression does not appear to be due to the heterogeneous distribution of cells through the cell cycle. Analogously, studies of the expression of these kinases in a variety of other tissues suggest that *Hunk* and *Punc* expression may also identify subsets of cells in other organs besides the breast. These observations suggest that *Hunk* and *Punc* are differentially expressed in distinct

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[6] H. P. Gardner, J. V. Rajan, S. T. Marquis, N. G. Copeland, D. J. Gilbert, N. A. Jenkins, and L. A. Chodosh, Cloning and chromosomal localization of a novel CaM kinase, *Punc*, manuscript in preparation.

[7] H. P. Gardner, S. I. Ha, S. T. Marquis, and L. A. Chodosh, Spatial and temporal regulation of *Hunk* expression during normal mammary gland development, manuscript in preparation.

[8] H. P. Gardner, S. I. Ha, and L. A. Chodosh, Differentiation-dependent expression of a novel calcium-calmodulin-dependent protein kinase, *Punc*, in the murine breast, manuscript in preparation.

epithelial cell subtypes in the breast that are differentially regulated during pregnancy.

To further investigate this hypothesis, we have examined *Hunk* and *Punc* expression in a panel of mammary epithelial cell lines derived from independent mammary adenocarcinomas arising in transgenic mice expressing the *neu,* c-*myc*, H-*ras*, or *int2* oncogenes.[7,8] Surprisingly, all eight cell lines derived from breast tumors that arose in transgenic mice expressing the *neu* or H-*ras* oncogenes were found to express high levels of *Hunk* mRNA, whereas none of the seven cell lines derived from breast tumors that arose in transgenic mice expressing the *c-myc* or *int-2* oncogenes expressed detectable levels of *Hunk*. Conversely, all seven cell lines derived from breast tumors that arose in transgenic mice expressing the *c-myc* or *int-2* oncogenes expressed *Punc* mRNA, whereas none of the eight cell lines derived from breast tumors that arose in transgenic mice expressing the *neu* or H-*ras* oncogenes expressed detectable levels of *Punc*. In each case, kinase expression levels observed in tumor cells were significantly higher than those observed in nontransformed mammary epithelial cells.

The heterogeneous spatial patterns of *Hunk* and *Punc* expression in the breast, along with the mutually exclusive patterns of expression of these two kinases in transgenic mammary epithelial cell lines, suggest that these novel serine-threonine kinases may be differentially expressed in distinct mammary epithelial cell subtypes that are themselves differentially regulated during pregnancy. The observation that *Hunk* and *Punc* are overexpressed in cell lines derived from breast cancers induced by the *neu* or c-*myc* oncogenes, respectively, suggests either that *Hunk* and *Punc* are downstream targets of the *neu* and c-*myc* oncogenes or that these kinases identify epithelial cell subtypes that are preferentially transformed either by *neu* or c-*myc*.

Each of these hypotheses is based on our observations suggesting that the normal mammary epithelium appears to be composed of distinct *Hunk*- and *Punc*-expressing cell types. The first hypothesis postulates that *Hunk* mRNA expression is activated by the *neu* and/or H-*ras* pathways, whereas *Punc* mRNA expression is activated by the c-*myc* and/or *int2* pathways. In this model, *neu* (or c-*myc*) transgene expression in the mammary epithelium induces *Hunk* (or *Punc*) expression in all mammary epithelial cell types that express the transgene. As a consequence, tumors that arise from the epithelium display the same differential pattern of expression exhibited by the parental normal transgenic mammary epithelium. The second hypothesis postulates that *neu* and c-*myc* preferentially transform two different mammary epithelial cell types, one of which (in the case of *neu*) is marked by *Hunk* expression and the other of which (in the case of *c-myc*) is marked by *Punc* expression. In this model, overexpression of *Hunk* in *neu*-induced tumors reflects the selection and outgrowth of an *Hunk*-expressing epithelial cell subtype that otherwise represents a minor fraction of cells in the normal mammary epithelium. That is, *Hunk* and *Punc* expression may be restricted to distinct epithelial cell subtypes that are preferentially transformed by these oncogenes.

Our data suggest that the novel serine/threonine kinases identified in our laboratory may serve as markers for biologically interesting subpopulations of epithelial cells in the breast that are relevant both to development and carcinogenesis. Current work in our laboratory on *Hunk*, *Punc*, and *Krct* focuses on placing these kinases in known or novel signal transduction pathways and on determining their role in mammary development and carcinogenesis using transgenic and knockout animal models as well as tissue culture model systems. In addition, we have cloned the human homologues for each of these genes and are currently determining whether *Hunk*, *Punc*, and *Krct* are mutated, amplified, or overexpressed in human tumors or tumor cell lines.

## Parity-induced Changes in the Breast

A third approach that our laboratory is taking to explore the relationship between development and carcinogenesis in the breast is to focus on the molecular and cellular changes that occur in the breast as a result of reproductive events known to influence breast cancer risk. Epidemiological studies have consistently shown that women who undergo an early first full-term pregnancy have a significantly reduced lifetime risk of breast cancer (1, 56–64). This association is independent of parity (*i.e.*, number of live births). In contrast, women who undergo their first full-term pregnancy after the age of 30–35 years appear to have a risk of breast cancer that is actually higher than that of nulliparous women. This suggests that parity-induced protection against breast cancer is principally dependent upon the timing of a first full-term pregnancy rather than on its occurrence *per se*. These observations imply that an early first full-term pregnancy results in a change in the breast, either directly or indirectly, that confers a decreased risk for the subsequent development of breast cancer. Because aborted pregnancies are not associated with a decreased risk for breast cancer, it has been hypothesized that the protective effect of parity requires attaining the terminally differentiated state of lactation (2, 3, 6, 59, 65–71). Unfortunately, the biological basis of parity-induced protection against breast cancer is unknown. In principle, the protective effect of early first childbirth could result from the pregnancy-driven terminal differentiation of a subpopulation of target cells at increased risk for carcinogenesis, from the preferential loss of a subpopulation of target cells during postlactational regression or from a permanent systemic endocrine change affecting the breast in such a way as to reduce the risk of carcinogenesis. Clearly, a more thorough elucidation of the molecular and cellular changes that take place in the breast as a result of parity will be required to fully understand this phenomenon.

The realization that specific reproductive endocrine events alter breast cancer risk in a predictable fashion raises the possibility that events known to decrease breast cancer risk might be mimicked pharmacologically. The desire to pursue this objective is heightened by the fact that, although it is now possible by genetic means to identify women who are at elevated risk for developing breast cancer, interventions between the extremes of more frequent mammographic screening and prophylactic bilateral mastectomy are only now beginning to be considered. As such, reducing breast cancer risk via hormonal manipulations designed to mimic naturally occurring endocrine events could represent a feasible alternative. It is to this end that both early first full-term pregnancy and early menopause have been proposed as logical paradigms on which to model the hormonal chemoprevention of breast cancer. The achievement of this goal, however, has been hampered by current ignorance regarding the mechanism by which reproductive history alters breast cancer risk. As such, the rational design of hormonal chemoprevention regimens would benefit from a better understanding of the influence of development on breast cancer risk. An additional stumbling block in the development of chemoprevention regimens aimed at reducing breast cancer risk has been the prolonged and costly clinical trials required to determine the efficacy of these regimens due to reliance on the development of breast cancer as a clinical end point (72–75). As such, the identification and use of intermediate molecular end points that accurately identify changes in the breast associated with changes in breast cancer risk would facilitate the development of such chemopreventive regimens. To this end, we have chosen to exploit the relationship between development and carcinogenesis in the breast to generate rational and biologically plausible candidate surrogate end point biomarkers.

The mechanism of parity-induced protection against breast cancer

is likely to involve complex genetic and epigenetic processes that may be influenced by reproductive endocrine variables as well as by inherited genotypes. In this context, it is useful to analyze complex processes such as this in model systems that recapitulate relevant epidemiological findings, permit critical aspects of reproductive history to be rigorously controlled, reduce genetic variation, and permit the examination of molecular and cellular events at defined developmental stages of interest in normal tissue. The use of animal models to study the impact of mammary gland development on breast cancer risk is facilitated by the fact that the structure, function, and developmental stages through which the mammary gland passes are similar in humans and in rodents (76, 77). Administration of the carcinogen DMBA to nulliparous Sprague Dawley rats induces mammary adenocarcinomas that are hormone dependent and histologically similar to human breast tumors. In contrast, rats that have previously undergone a full-term pregnancy are highly resistant to the induction of breast cancer by carcinogen administration, as compared with age-matched nulliparous controls (2, 6, 78–83).

Paralleling these functional differences, there are also marked morphological differences between the adult nulliparous mammary gland and the mammary glands of age-matched parous littermates that have undergone a single cycle of pregnancy, lactation, and regression. These parity-induced morphological changes are permanent because nulliparous and parous glands may be distinguished easily even after 1 year of postlactational regression (3).[9] Similar morphological changes are also seen in mice and in rats and are analogous to those reported in the parous human breast (70, 77). These observations support the hypothesis that parity results not only in a permanent change in the functional state of the breast (*i.e.*, susceptibility to carcinogenesis) but also in permanent structural changes in the breast. Finally, the fact that the Sprague Dawley DMBA model system mirrors complex epidemiological phenomena observed in humans, and that numerous molecules believed to play important roles in the pathogenesis of human breast cancer have similar effects in rodents, suggests that rodent model systems such as this can be a valuable tool for understanding fundamental aspects of mammary gland biology and breast cancer etiology.

We hypothesize that understanding the impact of parity on breast cancer risk will require a thorough understanding of the manner in which reproductive history affects subpopulations of cell types present in the breast. To address this hypothesis, we are using rodent model systems to identify and evaluate genes that are differentially expressed in the breast as a function of parity. Candidate genes that are specifically expressed in either the parous or the nulliparous rodent breast are being isolated and identified using a variety of approaches. These differentially expressed genes are being used as biomarkers for the cellular and molecular changes that occur in the breast as a result of an early first full-term pregnancy to define the impact of early parity on the development and differentiation of specific cell types in the breast. Finally, biomarkers that are found to be biologically informative in the rodent model system are being tested for their ability to detect parity-associated changes in histologically normal breast tissue obtained from nulliparous and parous women with known reproductive history and hormone exposures. The level and spatial pattern of expression of each of these candidate biomarkers is being analyzed in human tissue and evaluated with respect to parity as well as other parameters of reproductive endocrine history, such as age, age at first full-term pregnancy, menopausal status, and exogenous hormone use. These studies will determine whether candidate biomarkers characterized in rodent model systems can specifically detect parity-induced changes in the human breast.

To date, this approach has yielded a variety of genes that are expressed at higher levels in the mammary glands of parous animals as compared with age-matched virgin controls, confirming the utility of this approach for isolating genes that are specifically expressed in the breast as a function of reproductive history.[9] Several of the parity-specific genes that we have initially isolated are markers of mammary epithelial cell differentiation, such as milk proteins. This finding suggests that the parous breast is more "differentiated" than the nulliparous breast and, as such, is consistent with the proposal made by Russo and Russo (2, 84) that parity protects against breast cancer by virtue of the differentiation that it induces. The developmental patterns of expression of milk protein genes are notably heterogeneous because each is up-regulated at a specific point in the alveolar differentiation pathway (85). Interestingly, we have found that the expression patterns of several of these genes reflect subtle aspects of reproductive history.[9] As such, studying the regulated expression of this class of genes as a function of reproductive history may provide insights into parity-related events in the breast. In addition, we have isolated a number of genes that are as yet unidentified. Given their interesting developmental patterns of regulation and parity-specific pattern of expression, these genes appear to represent an informative pool of candidate biomarkers for detecting changes in the breast associated with reproductive events.

In theory, the parity-specific pattern of expression for a given biomarker could reflect a global increase in expression of the gene in all mammary epithelial cells, an increase in the percentage of expressing cells in the breast, or both. We are analyzing the developmental pattern of expression of candidate genes by *in situ* hybridization to distinguish between these mechanisms. Our results indicate that parity-specific patterns of expression for different genes result from distinct developmental pathways. For example, these studies reveal examples of parity-dependent global changes in expression as well as parity-dependent changes in the abundance of expressing cells. This latter example is suggestive of a permanent pregnancy-induced expansion in the number of cells expressing a given biomarker in the breast.[9] These findings are consistent with the hypothesis that reproductive events may permanently alter the biology of the breast by differentially affecting subpopulations of cells.

We have also determined the impact of several reproductive parameters on the differential pattern of expression of these genes.[9] These experiments reveal that the parity-specific pattern of expression for some genes is independent of age, duration of postlactational regression, and age at first full-term pregnancy. In contrast, other genes we have identified are expressed in a parity-specific manner in the mammary glands of animals that have been mated as adolescents but not in the mammary glands of animals that have been mated as adults. These results suggest that the regulation of expression of such genes reflects developmental events in the mammary gland that are specific for age at first full-term pregnancy. These findings suggest that candidate cDNA biomarkers generated by these approaches may provide insight into subtle aspects of the molecular and cellular changes that occur in the breast as a result of parity. Ultimately, these studies are intended to gain sufficient understanding of the molecular pathways responsible for parity-induced protection against breast cancer in order to permit this naturally occurring protective event to be mimicked pharmacologically.

## Summary

The current aims of this laboratory are designed to develop the molecular tools required to understand the relationship between nor-

[9] C. M. D'Cruz, J. Wang, S. I. Ha, and L. A. Chodosh, Reproductive history results in a permanent change in the expression of specific genes in the murine breast, manuscript in preparation.

mal mammary gland development and mammary carcinogenesis, as reflected in the epidemiology of reproductive endocrine risk factors for breast cancer. We have taken three approaches toward understanding this relationship, including: determining the role normally played by breast cancer susceptibility genes in mammary epithelial development; studying the function of three novel protein kinases in the breast; and identifying and analyzing genes that are specifically expressed in the breast during developmental stages associated with changes in breast cancer risk. We anticipate that these approaches will ultimately lead to a clearer understanding of the mechanisms by which breast cancer susceptibility is modulated by reproductive history.

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## Discussion

*Dr. Andrew Feinberg:* I have a really simple-minded question. These are very elegant studies, but I worry a bit about transgene-induced tumors, because in a sense you're starting with loaded dice. Aren't there any models of spontaneous mammary tumorigenesis? I thought there were some dogs or other species that developed cancer in a similar epidemiological manner as you mentioned for humans at the beginning. But, I don't know this field, so I may be totally wrong.

*Dr. Chodosh:* It is true that there are certain breeds of dogs that do develop breast cancer spontaneously, though I am not aware of any that show parity-induced protection against breast cancer. Obviously, how you choose a model to study a particular question is a central issue. Regarding transgenic rodent models of breast cancer there are a couple of points worth making.

The first is that our main experimental thrust is to look at the normal developmental biology of the breast. There is no question that the developmental stages through which the breast passes for both the mouse and the rat are exceedingly similar to what one finds in the human. That is, the developmental processes are as highly conserved as histology and tissue architecture.

The second is that it's quite clear from transgenesis experiments that many of the pathways that are altered during the process of carcinogenesis in the human breast cause similar problems in the rodent breast when altered by transgenic approaches. That is, the molecular pathways involved are highly conserved. So, while tumor development in a transgenic system is not "spontaneous" in the same way that we think of for human breast cancers, I would argue that the

history of cancer biology suggests that they are still quite useful models to examine pathways involved in development and carcinogenesis. So at the moment, as far as animals that we can work with, particularly those that we can genetically manipulate, we have mice. Similarly, in the rat, one is somewhat restricted to carcinogen-induced models, which may or may not faithfully mimic the processes involved in human carcinogenesis.

We think about the suitability of our model systems a great deal, and it's not clear to me that there's another *in vivo* system available at the present time that's more appropriate.

*Speaker:* Do you have any evidence these kinases play similar roles in the human breast? Because human breast cancer is quite different. Pathological studies are quite different from real breast cancer, because it's quite complicated by different pathways. So, my interest at the moment is that even if we are able to link these kinases to the set of human reactants, it is different with different types of breast cancer and different kinases being expressed. How do you plan to address these potential differences?

*Dr. Chodosh:* A very important question, which explains why we are moving into human tissue and human breast cancer cell lines to address some of these issues. This is information that we're currently gathering. The data that I showed you in human breast cancers and cancer cell lines are quite recent, so it's too preliminary for us to know whether there is some correlation between the expression of our kinases and Erb2 status or ER status, or a particular histological cell type. Regarding tumors that are marked by *Hunk* or *Punc* expression, clearly we would want to know whether they behave differently in terms of patient prognosis or response to therapy. We don't know that yet, though that's certainly something that we're very interested in.

*Dr. Robert Ryan:* I would like to ask, have you considered perhaps doing something like the chip-based assay where now you use the MMTV-neu and MMTV-c-myc breast cancer cell lines and test those samples for changes by looking at the various genes that are up-regulated or down-regulated. It might give you a handle on that, do you think?

*Dr. Chodosh:* Yes, that's certainly a possibility. In the context of DNA chip technology, I think we'd probably want to make the fewest possible changes that we could, starting with the most normal cells we can, then induce expression of a *Hunk* or *Punc* transgene and ask what genes are downstream, as opposed to using as a starting point tumor cell lines that obviously have undergone many unrelated changes over the long period of time they have been in culture. Certainly, I agree it's an important new technology.

# MYC induces mammary tumorigenesis by means of a preferred pathway involving spontaneous *Kras2* mutations

CELINA M. D'CRUZ[1], EDWARD J. GUNTHER[1,2], ROBERT B. BOXER[1], JENNIFER L. HARTMAN[1], LOUIS SINTASATH[1], SUSAN E. MOODY[1], JAMES D. COX[1], SEUNG I. HA[1], GEORGE K. BELKA[1], ALEXANDER GOLANT[1], ROBERT D. CARDIFF[4] & LEWIS A. CHODOSH[1,3]

[1]*Department of Molecular & Cellular Engineering,* [2]*Division of Hematology-Oncology,* [3]*Division of Endocrinology, Diabetes and Metabolism, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA* [4]*Center for Comparative Medicine, University of California at Davis, Davis, California, USA Correspondence should be addressed to L.A.C.; email: chodosh@mail.med.upenn.edu*

**Although the process of mammary tumorigenesis requires multiple genetic events, it is unclear to what extent carcinogenesis proceeds through a preferred secondary pathways following a specific initiating oncogenic event. Similarly, the extent to which established mammary tumors remain dependent on individual mutations for maintenance of the transformed state is unknown. Here we use the tetracycline regulatory system to conditionally express the human myelocytomatosis oncogene (*MYC*) in the mammary epithelium of transgenic mice. *MYC* encodes a transcription factor implicated in multiple human cancers. In particular, amplification and overexpression of MYC in human breast cancers is associated with poor prognosis, although the genetic mechanisms by which MYC promotes tumor progression are poorly understood[1,2]. We show that deregulated MYC expression in this inducible system results in the formation of invasive mammary adenocarcinomas, many of which fully regress following MYC deinduction. Nearly half of these tumors harbor spontaneous activating point mutations in the '*ras* family' of proto-oncogenes (not to be confused with the 'resistance to audiogenic seizures' gene) with a strong preference for *Kras2* compared with *Hras1*. Nearly all tumors lacking activating *ras* mutations fully regressed following MYC deinduction, whereas tumors bearing *ras* mutations did not, suggesting that secondary mutations in *ras* contribute to tumor progression. These findings demonstrate that MYC-induced mammary tumorigenesis proceeds through a preferred secondary oncogenic pathway involving *Kras2*.**

We mated transgenic mouse lines expressing the reverse tetracycline-dependent transcriptional activator (rtTA) under the control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR; mouse line designated MTB), to a second transgenic line containing exons 2 and 3 of human *MYC* under the control of the tetracycline-dependent minimal promoter (tetO; mouse line designated TOM)[3,4]. Recent analysis of female MTB mice demonstrated high levels of rtTA mRNA expression in the mammary gland and salivary gland, and little or no detectable expression in other tissues (E. Gunther, submitted manuscript). Administration of doxycycline to MTB/TOM, MTB, TOM and non-transgenic littermates resulted in high levels of *MYC* transgene expression only in MTB/TOM bitransgenic female mice (Fig. 1*a*). Mammary tissue from uninduced bitransgenic littermates showed no transgene expression indicating that this system is tightly regulated. Finally, ornithine decarboxylase (*Odc*) mRNA levels increased 2.2-fold, and growth arrest and DNA damage-inducible (*Gadd45a*) mRNA levels decreased 2.0-fold [AU: replace with appropriate percentage.], in induced bitransgenic females compared with uninduced controls demonstrating that the MYC transgene encodes a functional protein (Fig. 1*a* and data not shown [AU: Confusing. Please indicate what's in the fig. and what is not shown.])[5,6].

Morphological analysis showed that the mammary glands of MTB/TOM mice induced with doxycycline for 30 days were hyperplastic compared with either uninduced MTB/TOM or induced MTB control mice (Fig. 1*b*). Consistent with this, immunohistochemistry using antibodies specific for BrdU revealed a greater than 10-fold increase in BrdU incorporation in the mammary epithelium of induced bitransgenic females compared with control mice ($P < 0.001$) (Fig 1*b*). Similarly, TUNEL analysis of mammary tissue demonstrated a greater than 10-fold increase in TUNEL-positive epithelial cells in induced bitransgenic mice compared with controls ($P < 0.001$; Fig. 1*b*). Together these data indicate that MYC overexpression in the mammary epithelium increases both proliferation and apoptosis.

Compared with MTB/TOM mice induced with doxycycline for 30 days, the mammary glands of bitransgenic mice induced for 4 months displayed more severe morphological abnormalities, including hyperplastic, atypical lobuloalveolar growths referred to here as dysplasia (Fig. 1*c*). In contrast, uninduced bitransgenic mice maintained normal mammary gland morphology indistinguishable from that found in wild-type mice (Fig. 1*c* and data not shown [AU: Same problem.]). W next determined whether *MYC* transgene overexpression is required for the maintenance of hyperplastic lesions in MTB/TOM mice. We treated bitransgenic mice doxycycline for 30 weeks (chronically induced) and examined non-tumor-bearing glands from these mice 12 weeks after doxycycline withdrawal. Mammary glands from chronically induced bitransgenic mice had numerous epithelial hyperplasias and dysplasias, but most epithelial ducts from de-induced bitransgenic mice were histologically normal, indicating that hyperplastic and dysplastic mammary lesions that develop as a consequence of transgene overexpression remain dependent on MYC for their maintenance (Fig. 1*d*).

Consistent with the histological changes observed in chronically induced MTB/TOM mice, bitransgenic females developed mammary tumors with high penetrance (86%, $n = 57$) following an average of 22 weeks of induction (Fig. 2*a*). MTB mice chronically treated with doxycycline ($n = 15$), as well as untreated MTB

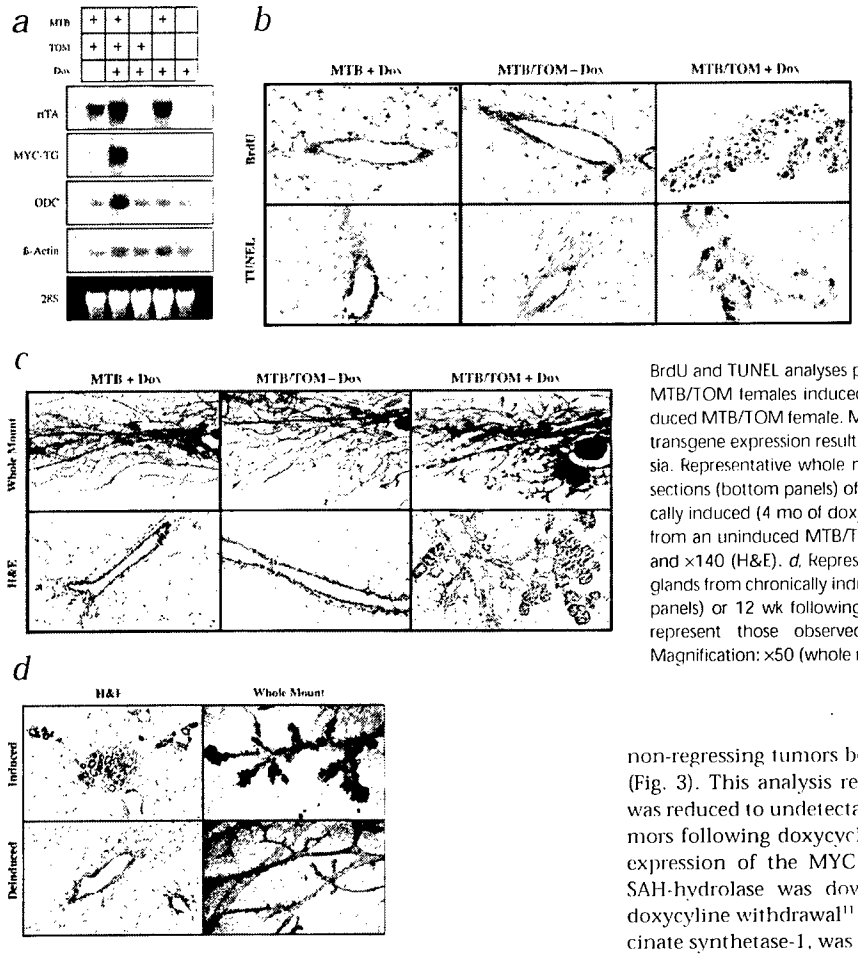


**Fig. 1** Chronic induction of MYC in the mammary epithelium of MTB/TOM mice results in reversible hyperplastic lesions. ***a*** Inducible transgene expression in MTB/TOM females is tightly regulated and encodes functional MYC. Duplicate northern blots containing mammary gland RNA from 8-wk-old female nulliparous FVB wild-type mice or age-matched mice bearing MTB, TOM, or MTB/TOM transgenes. Induced mice (Dox+) were administered doxycycline at 2 mg/ml in their drinking water for 30 d. ***b***, Induced MTB/TOM mice had elevated levels of mammary epithelial proliferation and apoptosis. Representative BrdU and TUNEL analyses performed on mammary sections from MTB and MTB/TOM females induced with doxycycline for 30 d and from an uninduced MTB/TOM female. Magnification: ×300. ***c***, Chronic induction of MYC transgene expression results in mammary epithelial hyperplasia and dysplasia. Representative whole mounts (top panels) and hematoxylin and H&E sections (bottom panels) of the #4 mammary gland harvested from chronically induced (4 mo of doxycycline) female MTB and MTB/TOM mice, and from an uninduced MTB/TOM control. Magnification: ×5 (whole mounts) and ×140 (H&E). *d*, Representative H&Es and whole mounts of mammary glands from chronically induced MTB/TOM mice either on doxycycline (top panels) or 12 wk following doxycycline removal (bottom panels). Results represent those observed in 19 mammary glands from 8 mice. Magnification: ×50 (whole mount) and ×300 (H&E)

($n$ = 20), TOM ($n$ = 18) and MTB/TOM ($n$ = 10) mice, exhibited normal histology of mammary tissues and did not develop tumors. Histological analysis of tumors from MTB/TOM females revealed invasive mammary adenocarcinomas similar to those previously described in MMTV-*Myc* transgenic mice[9,10] (Fig. 2*a*).

To determine whether MYC overexpression is required for the maintenance of mammary adenocarcinomas, we withdrew doxycycline from chronically induced, tumor-bearing MTB/TOM mice. In a subset of tumors, doxycycline withdrawal resulted in the rapid regression and clinical disappearance of invasive mammary adenocarcinomas (median time to disappearance was 14 d) indicating that maintenance of these tumors is dependent on *MYC* transgene overexpression (Fig. 2*b*). In contrast, other tumors continued to grow following doxycycline withdrawal or decreased in size to a variable extent and then resumed growth.

The fact that a subset of MYC-induced tumors grow in the absence of doxycycline indicates that additional genetic alterations have occurred that permit growth in the absence of MYC overexpression. Alternatively, the doxycycline-independent growth of such tumors could be due either to doxycycline-independent induction of the *MYC* transgene or to compensatory activation of MYC transcriptional targets by endogenous MYC. To investigate this, we compared gene expression levels for the *MYC* transgene, endogenous MYC and MYC transcriptional targets in non-regressing tumors before and after doxycycline withdrawal (Fig. 3). This analysis revealed that *MYC* transgene expression was reduced to undetectable levels in all eight non-regressing tumors following doxycycline withdrawal (Fig. 3). Concordantly, expression of the MYC transcriptional targets fibrillarin and SAH-hydrolase was downregulated to basal levels following doxycyline withdrawal[11] (Fig. 3). A third MYC target, arginosuccinate synthetase-1, was downregulated in 6 of 8 tumor samples following doxycycline withdrawal[12]. These findings indicate that the MYC pathway is downregulated in most, if not all, non-regressing tumors following doxycycline withdrawal. Endogeneous MYC expression levels increased following doxycycline withdrawal but were lower than induced levels of MYC transgene expression (Fig. 3). As MYC represses its own transcription[12], upregulation of endogenous *MYC* in this context is consistent with the overall decreased activation of the MYC pathway (Fig. 3). These data indicate that the failure of tumors to regress following doxycycline withdrawal does not result from failure of *MYC* transgene down-regulation, or from compensatory upregulation of the endogenous MYC pathway.

The long latency period and stochastic formation of tumors in MTB/TOM mice indicated that additional mutations were likely to be required for tumorigenesis. As forced co-overexpression of Hras1 and MYC in the mammary glands of transgenic mice has been shown to accelerate mammary tumorigenesis[13,14], we examined MYC-induced mammary tumors for spontaneous *Hras1* mutations. Exons 1 and 2 of *Hras1* were amplified and sequenced from mammary tumors arising in MTB/TOM mice. We detected no mutations in *Hras1* among 47 tumors. Since the *ras* family members, *Kras* and *Nras*, are also mutated in human cancers, we examined these genes for activating point mutations. We found 49% (23/47) of tumors to harbor activating point mutations in either codons 12 or 61 of *Kras2* or *Nras* (*Kras2* versus *Hras1*, $P = 1 \times 10^{-6}$; *Nras* versus *Hras1*, $P = 0.012$). Approximately three quar-

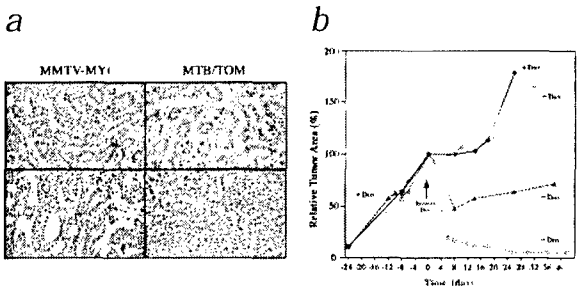


**Fig. 2** *MYC* transgene expression is required for maintenance of established mammary tumors. ***a***, Chronic induction of *MYC* transgene expression results in mammary adenocarcinomas. Hematoxylin and H&E sections of tumors from MMTV-*Myc* and induced MTB/TOM mice. Magnification: ×500. ***b***, Graph showing representative tumor regression patterns following transgene deinduction in three independent chronically induced MTB/TOM females (○,△ and ◇). A control tumor (◆) from a mouse maintained continuously on doxycycline is shown. One tumor (○) underwent full regression to a clinically undetectable state 25 d after doxycycline removal. A second tumor partially regressed during the first 10 d after doxycycline withdrawal, plateaued (△) then resumed growth. Growth of a third tumor (◇) showed essentially no regression

ters of *ras* mutations occurred in *Kras2* (17/23), with the remainder occurring in *Nras* (6/23) (*Kras2* versus *Nras*, $P = 0.008$). We detected no mutations in *Kras2*, *Hras1* or *Nras* in hyperplastic mammary glands from MTB/TOM mice induced with doxycycline for up to 60 days ($n = 9$) or in mammary glands from wild-type mice ($n = 5$).

To confirm these results, we examined mammary adenocarcinomas arising in MMTV-*Myc* transgenic mice for mutations in *ras* family members. This analysis revealed that 44% (8/18) of MYC-induced tumors harbored detectable *Kras2* mutations in codons 12 or 61, whereas we saw no mutations in *Hras1* or *Nras* (*Kras2* versus *Hras1* and *Kras2* versus *Nras*, $P < 0.001$). Thus, analysis of two independent mouse models for MYC-induced carcinogenesis strongly indicates that deregulated expression of MYC in the mammary gland selects for spontaneous activation of the *ras* pathway *in vivo*.

To investigate whether tumor regression in this model system is affected by the presence of *ras*-activating mutations, we determined the extent of tumor regression in a panel of 24 primary mammary adenocarcinomas, 15 of which had detectable *ras* mutations and 9 of which did not. We observed complete regression to a clinically undetectable state in 7 of 9 tumors lacking detectable *ras* mutations, but not in any of the 15 tumors bearing *ras* mutations

($\chi$-square, $P = 0.0005$). Three tumors bearing *ras* mutations showed essentially no regression, whereas the remaining twelve tumors bearing *ras* mutations exhibited partial regression, reached a plateau and then resumed growth. Two tumors lacking *ras* mutations did not regress fully. This might be due to failure to detect the existing *ras* mutations as a result of heterogeneity within the tumor, to activation of the *ras* pathway by other mechanisms or to alterations in other pathways. As the failure of MYC-induced tumors to regress following doxycycline withdrawal was not due to compensatory activation of MYC transcriptional targets by endogenous MYC, these data indicate that *ras* activation may abrogate tumor dependence on MYC overexpression for growth.

Here we have shown that deregulated expression of MYC in the mammary gland strongly selects for spontaneous activation of the *ras* pathway *in vivo* and that these activating mutations accompany the progression of tumors to a state that is no longer dependent on MYC for growth. This finding is the first example of spontaneous, recurrent activating mutations in a transgenic model for carcinogenesis. Inducible expressions of MYC or BCR-ABL in lymphoid tissues and of activated *Hras1* in melanocytes have been shown to result in tumors in transgenic mice, which are capable of regression when transgene expression is abrogated[15–17]. In contrast, reversible effects of oncogenes in epithelial cells *in vivo* have been described only in hyperplasias of the skin and salivary gland[18,19]. Long-term expression of T antigen in the salivary gland eventually results in hyperplasias that are not reversible, although the nature of the genetic changes responsible for this altered behavior is unclear[19]. We now extend these findings by showing that reversal of a single genetic lesion can be sufficient to reverse a common epithelial malignancy in the vast majority of cells, and by identifying a specific secondary mutation that accompanies the progression of tumors to a state independent of the initiating oncogenic stimulus

The observation that coexpression of MYC and *Hras1* can accelerate malignant transformation *in vitro* and *in vivo* constituted an early demonstration of synergy between oncogenic pathways[9,13,20]. However, although these studies proved that the synergistic action of MYC and activated *Hras1* is sufficient to accelerate the process of neoplasia, they did not address the question of whether the coexistence of such mutations represents a preferred pathway for tumorigenesis *in vivo*. Our finding that deregulated expression of MYC in the mammary gland strongly selects for spontaneous activating mutations in *ras* family members demonstrates that in the setting of a particular primary oncogenic stimulus, mammary tumorigenesis proceeds by

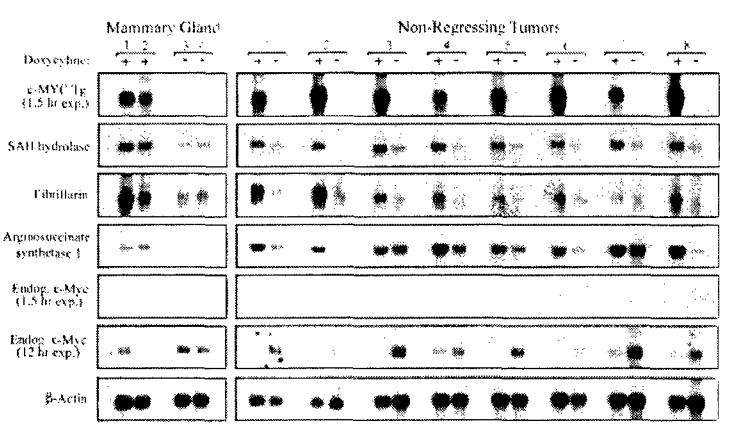


**Fig. 3** Non-regressing tumors downregulate MY pathways. Duplicate nothern blots containing either mammary tissue from doxycycline-induced or uninduced MTB/TOM mice or tumor tissue from doxycycline-induced or de-induced mice as indicated. Tissue from eight independent non-regressing tumors was collected before transgene de-induction by biopsy of actively growing tumors in mice on doxycycline (+), or in non-regressing tumors following doxycycline withdrawal (–)

the preferential activation of specific secondary oncogenic pathways.

The degree of specificity described here for the occurrence of spontaneous mutations in *Kras2* and *Nras*, as compared with *Hras1*, was unexpected given the previously demonstrated synergy between *Hras1* and MYC in mammary tumorigenesis in transgenic mice, and given the high degree of structural and functional homology among the proteins encoded by these genes. Our observations indicate that in the context of MYC overexpression in the mammary gland, activating mutations in *Hras1* and *Kras2* might not be functionally equivalent. This conclusion is consistent with the observation that particular *ras* family members are preferentially mutated in different types of human cancer, and by the recent demonstration of differences in the transcriptional targets of these genes[21,22]. Notably, activating mutations in *Kras2* have been reported in human primary breast cancers, though infrequently[23]. Potential explanations for the low level of *ras* mutations observed in human breast cancers are that the *ras* pathway is activated by other mechanisms or that such mutations are restricted to a particular subset of tumors. As such, our data indicate that it will be important to determine whether *Kras2* or *Nras* mutations are more common in breast cancers that have amplified the *MYC* locus.

Full regression of mammary adenocarcinomas in MTB/TOM mice strongly correlated with the absence of detectable *ras* mutations. This indicates that therapeutic targeting of MYC may be an effective treatment approach only for MYC-induced breast cancers in which the *ras* pathway has not been activated. As in other inducible transgenic model systems, a fraction of the MYC-induced tumors in our model system that had fully regressed following transgene de-induction recurred after several weeks or months. This indicates that tumor heterogeneity might permit the emergence of neoplastic cells that are no longer dependent upon the MYC transgene for growth. Our observations that MYC overexpression selects for spontaneous mutations in *ras* family members *in vivo*, and that *ras* mutations accompany tumor progression to a transgene-independent state, suggest that identifying specific sets of preferentially associated oncogenic mutations will facilitate targeting of the multiple synergistic pathways that contribute to neoplastic growth.

## Methods

**Transgenic** mice. We generated the pMMTV-rtTA expression vector by cloning the 1.1 kb *Eco*RI/*Bam*HI fragment encoding rtTA from pUHD172-1neo (ref. 3) into pBS-MMTV-pA, which consists of a plasmid BlueScript backbone (Stratagene, La Jolla, California), the MMTV-LTR upstream of the *Hras1* leader sequence and a multiple cloning site directly upstream of the SV40 splice site and polyadenylation signal (E.J. Gunther, submitted). The pTetO-MYC expression vector was generated by cloning exons 2 and 3 of human *MYC* from pSV7Humyc (ref. 4) into pTet-Splice (Gibco Brl, Life Technologies, Rockville, Maryland). For each construct, founder lines were generated by injecting linearized plasmid DNA into fertilized oocytes collected from superovulated FVB mice. Transgenic mice and littermate controls were administered doxycyline (0.5–2.0 mg/ml) in their drinking water. Mice were monitored twice per week for tumor formation. Calipers were used to measure tumor area in two dimensions. Mice bearing tumors were biopsied to obtain tumor tissue before doxycycline withdrawal. After approximately 1 wk, doxycycline was withdrawn from the drinking water of biopsied mice and tumors were monitored for regression behavior. First-strand cDNA was prepared from tumor material collected at biopsy and exons 1 and 2 of *Hras1*, *Kras2* and *Nras* were amplified by PCR and sequenced to detect point mutations.

**Northern-blot hybridization.** We performed total RNA isolation and northern hybridization as described[24] using 3 μg of total RNA from snap-frozen tumors or mammary tissue from which the lymph node in the number four mammary gland had been removed. Blots were hybridized with cDNA probes for a 360 bp fragment of rtTA (nt 1441–1800), exons 2 and 3 of human *MYC*, *ODC* (nt 725–1204), *FBL* (39–540; encoding fibrillarin), *SAHH* (249–801), *ASS* (45–918), exon 1 of mouse *Myc*, or *Actb*. MYC transgene and endogenous *MYC* were detected using probes of approximately equal specific activity to facilitate comparison of expression levels.

**Immunohistochemistry.** We injected mice with 1 mg BrdU per 20 g body weight 2 h before killing. Mammary gland #4 was extracted and fixed overnight in neutral buffered formalin, transferred to 70% ethanol and embedded in paraffin. 5 μm sections on ProbeOn Plus (Fisher [AU: Provide city, state]) slides were dewaxed in Xylene, then sequentially rehydrated in 100%, 95%, and 70% ethanol, followed by PBS. Sections were pretreated in 2 M HCl for 20 min at room temperature, washed in 0.1 M Borate buffer pH 8.5 × 2, and rinsed in PBS. BrdU immunohistochemistry was performed using the Vectastain Elite ABC Kit (Vector, Burlingame, California), rat antibody against BrdU IgG (Vector) and a secondary biotinylated rabbit anti body against rat IgG according to manufacturer's instructions. Sections were counterstained for 10 min in 0.5% (w/v) methyl green in 1.0 M NaOAc, pH 4.0.

We performed TUNEL analysis using the Apoptag Peroxidase Kit (Intergen, Purchase, New York) according to the manufacturer's instructions. Sections were pretreated in Proteinase K (20 μg/ml) for 15 min at room temperature, washed in de-ionized water twice for 2 min each, incubated in equilibration buffer, then incubated at 37 °C for 1 h with a 1:10 dilution of TdT enzyme in 1× reaction buffer. Reactions were terminated developed using anti-digoxigenin-alkaline phosphatase Fab fragments (BMB [AU: City, state]) and nitroblue tetrazolium chloride per manufacturer's instructions, and counterstained in methyl green. Images from BrdU and TUNEL sections were captured digitally and areas of positively stained and unstained nuclei were quantitated by color segmentation analysis using Image-Pro Plus software (Media Cybernetics, Silver Spring, Maryland). Quantitative analysis was performed on 4–8 fields per section consisting of approximately 2,500–10,000 cells.

**Whole mounts.** We spread #4 mammary glands on glass slides and fixed for 24 h in 10% neutral buffered formalin. Glands were placed in 70% ethanol for 15 min followed by 15 min in deionized water before staining in 0.05% carmine/0.12% aluminum potassium sulfate for 24–48 h. Glands were dehydrated sequentially in 70%, 90% and 100% ethanol for 10 min each and then cleared in toluene or methyl salicylate overnight. For histological analysis, mammary glands were fixed as above and transferred to 70% ethanol prior to paraffin embedding. 5 μm sections were cut and stained with hematoxylin and eosin.


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# Impaired DNA Damage Response in Cells Expressing an Exon 11-deleted Murine Brca1 Variant that Localizes to Nuclear Foci

L. Julie Huber[1], Thomas W. Yang[1], Christopher J. Sarkisian[1], Stephen R. Master[1],Chu-Xia Deng[2] and Lewis A. Chodosh[1,3]

[1]Department of Molecular & Cellular Engineering and
[3]Division of Endocrinology, Diabetes and Metabolism
612 Biomedical Research Building II/III
University of Pennsylvania School of Medicine
421 Curie Boulevard
Philadelphia, PA 19104-6160

[2]Genetics of Development and Disease, Branch, 10/9N105
National Institute of Diabetes, Digestive, and Kidney Diseases
National Institutes of Health
Bethesda, Maryland 20892

*To whom correspondence should be addressed. Tel: (215) 898-1321; Fax: (215) 573-6725; Email: chodosh@mail.med.upenn.edu

Running title: Impaired Rad51 response in *Brca1*$^{\Delta 11/\Delta 11}$ MEFS

## ABSTRACT

Germline mutations in *BRCA1* are the most common cause of familial breast cancer. Although the subcellular localization of the full length BRCA1 protein was initially controversial, it is now widely accepted that BRCA1 is a nuclear protein and localizes to discrete nuclear foci during S phase. This localization is consistent with the reported nuclear functions of BRCA1, which include transcription and DNA repair. In addition to full length BRCA1, both human and mouse cells express an alternatively spliced variant, BRCA1-Δ11; this isoform lacks all of exon 11 including a putative nuclear localization signal, and has been reported to reside in the cytoplasm based on transient transfection experiments. Surprisingly, despite its abundance the functional significance of this naturally occurring isoform has largely been ignored, at least in part because its reported cytoplasmic localization would ostensibly preclude it from playing a role in nuclear processes. However, the finding that murine embryos bearing homozygous deletions of only the exon 11 region of *Brca1* survive longer than embryos that are homozygous for null alleles of *Brca1* suggests that exon 11-deleted isoforms may perform at least some of the functions of Brca1. In order to resolve this apparent inconsistency, and to address the functions of exon 11-deleted isoforms of Brca1, we have generated antisera to murine Brca1 and have analyzed cells derived from a murine model in which only the exon 11-deleted Brca1 isoform is expressed. Our results demonstrate that mouse Brca1 is identical to human BRCA1 with respect to its cell cycle regulation, DNA damage-induced phosphorylation, nuclear localization, and association with Rad51. Surprisingly, we show that endogenous exon 11-deleted isoforms of Brca1 localize to discrete nuclear foci indistinguishable from those found in wild type cells, despite the fact that they lack previously defined nuclear localization signals encoded in exon 11. However, we show here that unlike full length Brca1, exon 11–deleted Brca1 is not phosphorylated in response to DNA damage, and that Rad51 foci formation is significantly reduced in response to γ-irradiation in cells expressing only the exon 11–deleted isoform.

## INTRODUCTION

Germline mutations in *BRCA1* predispose women to early onset breast and ovarian cancer (17, 36). The *BRCA1* gene is composed of 23 exons that encode an 1863 amino acid full length protein, over half of which is encoded by an unusually large exon, exon 11, which is 3.4 kb in length. In addition to full length BRCA1 protein, $p220^{BRCA1}$, human cells contain alternatively spliced variants referred to as BRCA1-Δ11 (referred to here as $p97^{BRCA1}$) and BRCA1-Δ11b (referrred to here as $p110^{BRCA1}$) that lack all or most of exon 11, respectively (50, 53). These isoforms arise from in-frame splicing events and retain the highly conserved amino-terminal RING finger and carboxyl-terminal BRCT domains found in full length BRCA1, but lack the nuclear localization signals previously identified in exon 11 (10, 50, 53). The abundant expression of $p97^{BRCA1}$ and $p110^{BRCA1}$ has been demonstrated in a variety of adult tissues, including the human mammary gland, in which transcripts encoding $p110^{BRCA1}$ are expressed at levels comparable to those encoding $p220^{BRCA1}$ (31, 50, 53).

The observations that human BRCA1 is phosphorylated in response to ultraviolet light, ionizing radiation, and other agents that damage DNA, and the identification of BRCA1-interacting proteins such as RAD51 and RAD50-Mre11-p95 complexes that co-localize with BRCA1 following DNA damage, have suggested a role for BRCA1 in DNA repair (45, 51, 60). Subsequent experiments have confirmed this suggestion by demonstrating that human and mouse Brca1 are required for the repair of double-stranded DNA breaks (35, 47). BRCA1 has also been implicated in transcriptional regulation through the ability of its carboxyl-terminal domain to stimulate transcription in a variety of functional assays as well as by virtue of its demonstrated interaction with the nuclear proteins p53, pRB, CtIP, CBP/p300, ATF1, and RNA polymerase II holoenzyme complexes (2, 3, 9, 21, 28, 33, 37, 41-43, 58, 59). In addition, the recent finding that BRCA1 is a component of a SWI/SNF-related complex suggests that BRCA1 may play a role in coordinating processes such as repair and transcription through the remodeling of chromatin (6).

Initial reports describing the subcellular localization of BRCA1 were highly controversial. BRCA1 has been reported by different groups to localize to the cytoplasm, to the nucleus, to cytoplasmic tube-like invaginations in the nucleus, or to be secreted (12, 14, 26, 46). These reports

preceeded experiments demonstrating functional roles for BRCA1 in DNA damage and transcription, each of which would have suggested that BRCA1 was likely to reside in the nucleus. Indeed, the subsequent observation that BRCA1 compartmentalizes to nuclear foci during S phase and undergoes a DNA damage-dependent dynamic redistribution served to focus efforts on experiments designed to identify a nuclear role for BRCA1 (44).

In contrast to BRCA1, the properties and functions of the exon 11-deleted isoforms of BRCA1 are largely unknown. Previous experiments suggesting that BRCA1-Δ11 is localized to the cytoplasm were based on transient transfection protocols (50). However, the fact that similar approaches indicated a cytoplasmic localization for $p220^{BRCA1}$ suggested that determining the localization of exon 11-deleted isoforms would require examination of their endogenous expression patterns (53). Inconclusive results have been obtained regarding the cellular localization of $p110^{BRCA1}$; biochemical fractionation of transiently transfected cells has shown that $p110^{BRCA1}$ is distributed equally between nuclear and cytoplasmic fractions whereas immunofluorescence analysis of the same ectopically expressed protein was reported to yield exclusively cytoplasmic staining (53). These reports appear to be at odds with studies making use of BRCA1 antibodies that recognize determinants shared by full length BRCA1 and its isoforms, since these studies have generally failed to reveal the presence of BRCA1 proteins in the cytoplasm (46).

The finding that murine embryos bearing targeted mutations that abolish expression of full length Brca1, but leave Brca1-Δ11 expression intact, survive significantly longer than mice bearing targeted mutations that abolish expression of both Brca1 and Brca1-Δ11 suggests that in mouse cells, Brca1-Δ11 is able to partially compensate for the functions of full length Brca1 (16, 19, 22, 29, 30, 48, 56). Despite the decreased severity of their embryonic phenotype, embryonic cells derived from mice engineered to express only Brca1-Δ11 exhibit hypersensitivity to γ-irradiation, defective G2-M checkpoint function, centrosome amplification, and genomic instability (19, 48, 56). Furthermore, mice bearing mammary-specific deletions of exon 11 develop mammary adenocarcinomas with chromosomal instability (55). These data suggest that while Brca1-Δ11 may partially compensate for

Brca1 function during embryogenesis, this naturally occuring isoform lacks the ability to maintain genomic stability and suppress tumorigenesis.

In this report we demonstrate by biochemical fractionation and immunofluorescence that full length and exon 11-deleted isoforms of murine Brca1 are cell cycle regulated and compartmentalize to nuclear foci during S phase. In contrast to full length Brca1, we show that Brca1- Δ11 is not phosphorylated in response to DNA damage, is deficient in its ability to bind to Rad51, and is unable to promote the efficient formation of Rad51 foci. Taken together, these data suggest that Brca1-Δ11 may provide some of the functions of full length Brca1 during murine embryogenesis, but is unable to fully supplant the functions of full length Brca1 in the response to DNA damage.

## MATERIALS AND METHODS

**Generation of Antisera.** Regions encompassing amino acids 69-278 (mAb1), 809-1062 (mAb2), 995-1244 (mAb3), and 1365-1609 (mAb4) of the murine *Brca1* cDNA were subcloned into pGEX-6P-1 (Pharmacia). Lysates from E. coli transformed with these constructs were passed over a glutathione-Sepharose column and recombinant Brca1 protein was cleaved from the GST polypeptide with PreScission Protease according to the manufacturers instructions (Pharmacia). Antisera to purified Brca1 polypeptides were raised in rabbits (Cocalico Biologicals) and were affinity purified according to published methods (23).

**Cell Culture, Synchronization, and Fractionation.** HCll cells were grown in RPMI medium containing 10% bovine calf serum, 5 μg/ml insulin (Sigma), 10 ng/ml epidermal growth factor (Sigma), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Mouse embryo fibroblasts were grown in DMEM containing 15% fetal bovine serum supplemented with 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. 293T cells were grown in DMEM containing 10% bovine calf serum supplemented with 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. HCll cells and fibroblasts were serum starved at 75% confluency and refed with regular growth media 48 hours later. Fractionation was performed according to the manufacturer's instructions with the NE-PER kit (Pierce).

**Northern Analysis, Immunoblotting, and Immunoprecipitation.** Northern hybridization was performed as described using PCR probes encompassing nucleotides 2541-3298 within exon 11 and nucleotides 4827-5354 within the carboxyl terminus of *Brca1* (39). Cell lysates for immunoblotting were prepared in 50 mM Tris pH 8.0, 120 mM NaCl, 0.05% Nonidet P-40 (NP-40) with 100 μg/ml Pefabloc (BMB), 20 μg/ml aprotinin, 10 μg/ml leupeptin, 0.1 mM β-glycerophosphate, 50 mM NaF, and 1 mM sodium orthovanadate. Samples were routinely loaded onto 7% SDS-PAGE gels with the exception of experiments designed to detect changes in the mobility of Brca1, for which 5 or 6% gels

were run for extended periods. Wet transfer to nitrocellulose was performed overnight in buffer containing 192 mM glycine, 25 mM Tris base, and 20% methanol. Membranes were blocked for 1 hr in phosphate buffered saline containing 5% nonfat dried milk and 0.5% NP-40. RAD51 Ab-1, (Calbiochem), RAD50 Clone 13, (Transduction Laboratories), and Cyclin A H-432, (Santa-Cruz Biotechnology) antibodies were each used at a 1:1000 dilution in blocking buffer for 1 hr. β-tubulin antibody N-357, (Amersham) was used at a 1:40,000 dilution. A peroxidase-conjugated goat anti-rabbit IgG secondary antibody H+L, (Jackson Immunoresearch) was used at a 1:3000 dilution. Immunoprecipitations were performed for 1 hour at 4°C. Rad51 antibodies Ab-1 (Oncogene Research), I-20 and C-20 (Santa-Cruz Biotechnology), and affinity purified Brca1 antibodies were employed at 2 μg/ml. Immune complexes were precipitated with 20 μl of protein-A Sepharose and were washed 5 times with lysis buffer prior to the addition of 1X Laemmli sample buffer.

**Treatment with DNA Damaging Agents and Orthophosphate Labeling.** Gamma irradiation was administered using a CIS bio international (IBL 437c) source. Ultraviolet (UV) doses were administered using a Stratalinker (Stratagene). Hydroxyurea (HU; Sigma) was used at a final concentration of 1 mM. Cells were lysed 1 hr following treatment with genotoxic agents. For *in vivo* labeling experiments, irradiated cells were incubated with 5 mCi of $^{32}P$ orthophosphate in serum free medium immediately following dosing for 1 hr prior to lysis and immunoprecipitation.

**Immunofluorescence Analysis.** Cells were fixed and permeabilized according to published protocols (44). Affinity purified Brca1 antisera were used at a concentration of 2 μg/ml. RAD51 (Ab-1) antisera were used at a 1:1000 dilution. TRITC-conjugated secondary antibody (Jackson Immunoresearch) was used at a dilution of 1:250. All images were obtained by laser scanning confocal microscopy.

## RESULTS

**Characterization of Mouse Brca1 Antisera.** Immunoblotting analysis of HCll murine mammary epithelial cell extracts using murine Brca1 antibodies mAb1, mAb2, mAb3, and mAb4, identified a specific band that migrated at a predicted molecular weight of 210 kDa not recognized by preimmune sera (data not shown). To confirm that the antibodies recognize bona fide mouse Brca1, 293T cells were transfected with a mouse *Brca1* cDNA and lysates were prepared for immunoblotting. These studies revealed that polyclonal antibodies mAb1, mAb2, mAb3, and mAb4 each recognize a specific band at the predicted molecular weight for mouse Brca1 in extracts of *Brca1*-transfected 293T cells that were subjected to immunoblotting (Fig. 1A and data not shown).

To determine if mAb1 could specifically recognize endogenous Brca1, extracts from wild-type mouse embryo fibroblasts (MEFs) and from MEFs derived from mice harboring a germline deletion of the exon 11 region of Brca1 were analyzed by immunoblotting (48). *Brca1*$^{\Delta 11/\Delta 11}$ MEFs express a murine isoform of Brca1 analogous to the naturally occurring human BRCA1 variant encoding p97$^{BRCA1}$. Northern analysis with a probe that encompasses nucleotides 4827-5354 predicted to recognize both the full length and exon 11-deleted Brca1 transcripts was performed. As expected, a 3.9 kB transcript was detected in *Brca1*$^{\Delta 11/\Delta 11}$ cells and a 7.2 kB transcript was detected in cells that express p210$^{Brca1}$ (Fig.1C, left panel). A similar analysis with a probe encompassing nucleotides 2541-3298 within exon 11 detected only the full length Brca1 transcript (Fig.1B, right panel). Accordingly, extracts prepared from wild type MEFs revealed the presence of p210$^{Brca1}$ whereas extracts prepared from *Brca1*$^{\Delta 11/\Delta 11}$ MEFS did not, confirming that the 210 kDa polypeptide recognized by mAb1 is indeed Brca1 (Fig. 1C). In order to determine if the putative protein encoded by the exon 11-deleted transcript is present, immunoblotting analysis was performed on extracts derived from *Brca1*$^{\Delta 11/\Delta 11}$ MEFS. A major band of the predicted molecular weight, referred to here as p92$^{Brca1}$, was recognized by mAb1 antisera (Fig. 1D). This Brca1 isoform was also detected in embryonic brain extracts prepared from embryos heterozygous for the exon 11-deleted allele of

*Brca1*, as well as in extracts of testis and brain derived from wild type mice. These findings demonstrate that $p92^{Brca1}$ is a naturally occurring isoform of Brca1 (Fig 1E).

**Full length Murine $p210^{Brca1}$ and $p92^{Brca1}$ are Cell Cycle Regulated.** Previous experiments have shown that murine *Brca1* mRNA expression is regulated in a cell cycle-dependent manner with maximal levels of *Brca1* occurring during S phase of the cell cycle (39). Human *BRCA1* mRNA and protein share this cell cycle-dependent pattern of expression consistent with a conserved S phase-specific function for the human and mouse Brca1 proteins (11, 20, 40, 52). In order to determine if the protein expression pattern of Brca1 parallels that of its mRNA, synchronization experiments were performed using HCll murine mammary epithelial cells. As shown in Fig. 2A, the mouse Brca1 protein, $p210^{Brca1}$, is undetectable in serum starved cells and becomes apparent when cells have progressed into the G1 phase of the cell cycle approximately 8 hours following addition of serum containing media. Parallel experiments performed on $Brca1^{\Delta 11/\Delta 11}$ fibroblasts demonstrated that the cell cycle-dependent expression pattern of $p92^{Brca1}$ closely mimics that of $p210^{Brca1}$. Moreover, the temporal profile of $p92^{Brca1}$ and $p210^{Brca1}$ expression is similar to that of Rad51 and Cyclin A, but contrasts with that of Rad50 which is expressed at relatively constant levels throughout the cell cycle.

**Phosphorylation of $p92^{Brca1}$ Does Not Occur in Response to DNA Damage.** The phosphorylation of human $p220^{BRCA1}$ following treatment of cells with DNA damaging agents was an early indication that human BRCA1 is involved in a DNA damage response pathway. Similarly, immunoblotting analysis of lysates generated from HCll cells one hour following treatment with UV, $\gamma$-radiation, or HU revealed a dose-dependent shift in the migration of full length murine Brca1 by SDS-PAGE (Fig. 3A, top panel). A complete shift of $p210^{Brca1}$ similar to that described for human BRCA1 occurred in cells treated with 50 Gy .

$^{32}$P-orthophosphate labeling of HCll cells following treatment with 5 Gy demonstrated an increase in Brca1 labeling consistent with the supposition that, similar to human BRCA1, the

observed mobility shift is due to phosphorylation (Fig. 3B). Exposure of cells to $^{32}$P-orthophosphate has previously been shown to cause an increase in phosphorylation of human BRCA1 (51). The basal levels of phosphorylation observed in unirradiated HC11 cells may be due either to the activation of a DNA damage response pathway by $^{32}$P itself, or to cell cycle-dependent phosphorylation of Brca1. Significantly, a shift in p92$^{Brca1}$ was not observed in response to identical treatments with DNA damaging agents suggesting that this isoform may not be phosphorylated under these conditions (Fig 3A, bottom panel). This possibility was confirmed by $^{32}$P-orthophosphate labeling experiments in which increased phosphorylation of p92$^{Brca1}$ was not detected following treatment of cells with 20-50 Gy (Fig 3B, bottom panel and data not shown).

**Murine p210$^{Brca1}$ and p92$^{Brca1}$ Localize to Nuclear Foci.** In order to determine if the lack of phosphorylation of p92$^{Brca1}$ is due to aberrant cellular localization, biochemical fractionation of exponentially growing Brca1$^{\Delta 11/\Delta 11}$ fibroblasts was performed and nuclear and cytoplasmic fractions were analyzed by immunoblot analysis. To confirm the purity of these fractions, blots were probed with antisera specific either for β-tubulin or RAD50 as controls for cytoplasmic and nuclear proteins, respectively. Surprisingly, these studies revealed that p92$^{Brca1}$ was present in the nuclear fraction (Fig 4A). In order to confirm these results and to establish the subnuclear localization of p210$^{Brca1}$ and p92$^{Brca1}$, immunofluorescence analysis (IF) was performed (Fig. 4C) since localization to nuclear foci during S phase is a cardinal feature of human BRCA1 (44). Four independent antisera (mAb1-mAb4) revealed that p210$^{Brca1}$ localizes to nuclear foci during S phase in both mammary epithelial cell and WT MEFs. Strikingly, when similar experiments were performed on *Brca1*$^{\Delta 11/\Delta 11}$ fibroblasts using antisera directed against epitopes outside of exon 11, distinct nuclear foci were observed that were indistinguishable from those observed in HCll mammary epithelial cells and in wild type mouse embryo fibroblasts (Fig. 4C). Since *Brca1*$^{\Delta 11/\Delta 11}$ MEFs do not express p210$^{Brca1}$, we reasoned that any specific signal would be due to p92$^{Brca1}$. Consistent with this supposition, nuclear foci were not detected following IF using the exon 11-specific antisera mAb2 and mAb3. Notably, no signal was observed in the cytoplasm of HC11, WT MEFs, or *Brca1*$^{\Delta 11/\Delta 11}$ MEFs using any of the above antisera.

**Association of Rad51 with p92$^{Brca1}$ and Rad51 focus formation are compromised in *Brca1*$^{\Delta 11/\Delta 11}$ cells.** The exon 11 region of human BRCA1 protein has been shown to be required for binding to RAD51. This observation suggested the possibility that p92$^{Brca1}$ may not associate with Rad51 in *Brca1*$^{\Delta 11/\Delta 11}$ cells. In order to address this question p92$^{Brca1}$ was immunoprecipitated from extracts of *Brca1*$^{\Delta 11/\Delta 11}$ MEFs and analyzed by Western blotting with Rad51. Immunoblotting analysis revealed that Rad51 was readily detected in extracts derived from HC11 cells in which mAb1 and mAb4 had been used to immunoprecipitate p210$^{Brca1}$ (Fig. 5). In contrast, Rad51 was not detected in extracts derived from *Brca1*$^{\Delta 11/\Delta 11}$ MEFs that had been processed in an identical manner. In reciprocal coimmunoprecipitation experiments, p210$^{Brca1}$ was detected in HC11 extracts immunoprecipitated with Rad51 antisera. However, it was not possible to determine if p92$^{Brca1}$ was present in Rad51 immunoprecipitates due to the presence of a cross-reacting band that comigrated with p92$^{Brca1}$ (data not shown).

Rad51 has been shown to localize to subnuclear foci following treatment of cells with agents that induce double-stranded breaks (38). The lack of association of p92$^{Brca1}$ with Rad51 prompted us to examine Rad51 focus formation in WT and *Brca1*$^{\Delta 11/\Delta 11}$ MEFs. Three hours following irradiation with either 5 or10 Gy, Rad51 focus formation was assessed in cycling WT and *Brca1*$^{\Delta 11/\Delta 11}$ MEFs by IF (Fig. 6). An average of 29 and 45 Rad51 foci were detected in WT cells treated with 5 and 10 Gy, respectively, whereas an average of only 5 and 9 foci per cell were detected following similar treatement in *Brca1*$^{\Delta 11/\Delta 11}$ cells (Fig. 6A and B). This impaired response was not due to decreased levels of Rad51 in *Brca1*$^{\Delta 11/\Delta 11}$ MEFs as demonstrated by Western analysis of extracts from cells that had been treated in an identical manner as for immunofluorescence (Fig.6C). Notably, the absence of p210$^{Brca1}$ does not affect the previously demonstrated S phase-dependent expression of Rad51, suggesting that the inability to form foci is not due to aberrant cell cycle expression of Rad51 in *Brca1*$^{\Delta 11/\Delta 11}$ cells. (Fig.2B) (18, 49, 57). In aggregate, these data suggest that p92$^{Brca1}$ has a diminished ability to associate with Rad51, and that Rad51 focus formation is impaired in *Brca1*$^{\Delta 11/\Delta 11}$ MEFs.

## DISCUSSION

While human BRCA1 has been extensively characterized, little is currently known about its murine counterpart. In fact, the mouse Brca1 protein shares only 58% sequence identity to human BRCA1, a finding that has contributed to the suggestion that these proteins may have different functions(1, 7).In this report we present the first characterization of the mouse Brca1 protein and demonstrate that multiple features of the regulation, localization, and interactions of the mouse and human Brca1 proteins are conserved. Similar to its human homolog, mouse Brca1 is cell cycle regulated and localizes to nuclear foci during S phase. In addition, mouse Brca1 is phosphorylated in a dose-dependent manner in response to genotoxic agents suggesting that a similar kinase(s) exists in human and murine cells that is upstream of Brca1 in a DNA damage response pathway. Like human BRCA1, murine Brca1 also forms a complex with Rad51 consistent with experiments demonstrating that mouse Brca1 functions in the repair of double-stranded breaks by homologous recombination(35). In aggregate, these data validate the use of mouse models to study BRCA1 function in human cells.

We have analyzed the expression of a naturally occurring Brca1 isoform in fibroblasts derived from mouse embryos in which the exon 11 region of Brca1 has been specifically deleted. Strikingly, we have found that $p92^{Brca1}$ is localized to nuclear foci. This finding is consistent with our biochemical fractionation studies revealing that endogenous $p92^{Brca1}$ is present in the nucleus, as well as with previous findings in human cells that anti-BRCA1 antibodies do not appear to detect cytoplasmic BRCA1 staining, despite the fact that $p97^{BRCA1}$ and $p110^{BRCA1}$ would otherwise be expected to be localized there. Given the overlap in properties of $p92^{Brca1}$ and $p210^{Brca1}$, our finding that exon 11-deleted isoforms of Brca1 are also present in the nucleus raises for the first time the possibility that this isoform may partially compensate for mutations affecting Brca1, and may possess additional nuclear functions that are as of yet unrecognized.

Notably, our findings contrast with the cytoplasmic localization previously reported for human $p97^{BRCA1}$ and $p110^{BRCA1}$, each of which lack the nuclear localization sequences reportedly required for nuclear transport of $p220^{BRCA1}$ (50). Nevertheless, the reported partial nuclear localization

of human p110$^{BRCA1}$ suggests that sequences other than the canonical *BRCA1* nuclear localization sequences can be utilized for transport into the nucleus, or that exon 11-deleted isoforms of Brca1 could be transported to the nucleus via binding to other nuclear proteins (13, 24, 34). Such cryptic nuclear localization sequences may also be responsible for the nuclear localization of p92$^{Brca1}$. Alternatively, the difference in localization between the mouse and human isoforms may be due to either cell type-specific differences, species-specific differences, or the nature of the assays employed for these studies. We favor the latter hypothesis. Whereas studies in human cells determined the localization of exogenously expressed p97$^{BRCA1}$ and p110$^{BRCA1}$ using transient transfection assays, we have determined the localization of the endogenous Brca1 proteins. In this regard, previous reports have shown that the high levels of expression characteristic of transient transfection experiments may lead to mislocalization of BRCA1 to the cytoplasm (53). Nevertheless, we cannot rule out the possibility that p92$^{Brca1}$ may localize to the cytoplasm in cell types other than those examined here, or that human and mouse exon 11-deleted isoforms may localize differently.

Significantly, p210$^{Brca1}$ displays a mobility shift indicative of phosphorylation in response to DNA damage, whereas p92$^{Brca1}$ does not. Consistent with this, $^{32}$P-labeling experiments failed to reveal an increase in phosphate incorporation in p92$^{Brca1}$ in response to γ-irradiation suggesting that the inability to detect a shift is not due to a conformation of p92$^{Brca1}$ that precludes altered mobility by SDS-PAGE. Of note, the Cds1 kinase has been shown to phosphorylate serine 988 of human BRCA1 in response to γ-irradiation (27). A putative Cds1 phosphorylation site is also present in mouse Brca1 within the exon 11 region suggesting that p92$^{Brca1}$ may not be a target of Cds1. Moreover, a shift in the mobility of Cds1 protein on SDS-PAGE, which has been shown to correlate with kinase activation, occurs in both WT MEFs and Brca1$^{\Delta 11/\Delta 11}$ MEFs following irradiation suggesting that the absence of p92$^{Brca1}$ phosphorylation is not the result of an inactive Cds1 kinase (data not shown)(5, 8, 32). Following high dose γ-radiation, serines 1423 and 1524 of human BRCA1 are phosphorylated by the ATM kinase (15). Although these potential ATM phosphorylation sites are present in p92$^{Brca1}$, the absence of an observed increase in p92$^{Brca1}$ phosphate incorporation following γ-irradiation suggests that these sites do not undergo an increase

in phosphorylation. A potential explanation for these data is that ATM phosphorylation of Brca1 in response to DNA damage could be dependent on initial phosphorylation of serine 988 by Cds1. Alternatively, the exon 11 region may be necessary for Cds1 or ATM binding to Brca1 and this interaction may in turn be required for phosphorylation.

Our inability to detect a stable association between $p92^{Brca1}$ and Rad51 in *Brca1*$^{\Delta 11/\Delta 11}$ MEFS is consistent with results demonstrating that RAD51 binds to the exon 11 region of human BRCA1 (45). We now provide evidence that this interaction may be required for the efficient formation of Rad51 foci in response to γ-irradiation, a finding that is consistent with recent evidence that Rad51 foci are reduced in embryonic stem cells harboring a similar *Brca1* mutation (4). These data suggest that the inability to relocalize Rad51 may compromise the capacity of these cells to repair double-stranded breaks, thereby contributing to the defective G2/M checkpoint observed in response to ionizing radiation in these cells (56). In this regard, the recent demonstration that BRG-1, a component of the SWI/SNF complex, interacts with human BRCA1 through the exon 11 region is particularly intriguing in that it suggests a model in which the chromatin-remodeling function of BRCA1 may be associated with its ability to mediate the proper assembly of RAD51 (6).

Despite the shared properties of Brca1 and Brca1-Δ11, which suggest that exon 11-deleted isoforms may have nuclear functions, mouse knockout models clearly indicate that significant functional differences exist between full length and exon 11-deleted isoforms of Brca1. Foremost, mice engineered to express only Brca1-Δ11 are not viable, and embryonic cells derived from these mice demonstrate hypersensitivity to γ-irradiation, defective G2-M checkpoint function, centrosome amplification, and genomic instability (19, 48, 56). Moreover, cre-mediated excision of exon 11 in epithelial cells of the murine mammary gland leads to abnormal ductal morphogenesis and tumor formation (55). These experiments demonstrate that the exon 11 region is critical for normal Brca1 function. In this context, our data suggest that the inability of Brca1-Δ11 to provide G2-M checkpoint function, maintain genomic stability, and suppress tumorigenesis is not due to an inability of Brca1-Δ11 to be transported to the nucleus, to localize to nuclear foci, or to be cell cycle regulated, but rather may be related to the inability of $p92^{Brca1}$ to associate with Rad51 or other

proteins such as BRG-1 and Rad50 (60). In this regard, our data demonstrating that $p92^{Brca1}$ is not phosphorylated in response to DNA damage suggest that the signal transduction pathways activated by the replication checkpoint and by lesions caused by UV-irradiation do not converge on the $p92^{Brca1}$ protein. As such, deletion of exon 11 of BRCA1 appears to impair its DNA damage-dependent phosphorylation, which may in turn affect the localization to nuclear foci or function of BRCA1-interacting proteins such as BARD1, BRCA2, or the RAD50/MRE11/NBS complex.

The naturally occurring expression of Brca1-Δ11 during murine embrogenesis and in adult tissues suggests that exon 11-deleted isoforms may function in a variety of tissues. Moreover, in spite of a defect in γ-radiation-induced Rad51 focus formation in cells lacking full length Brca1, analysis of *Brca1*$^{\Delta 11/\Delta 11}$ embryos indicates that $p92^{Brca1}$ partially compensates for the lack of full length Brca1 during murine embryogenesis. The most striking evidence for this conclusion is the postnatal survival of targeted mouse lines in which only the $p92^{Brca1}$ protein is predicted to be expressed (16). Presumably this is due to interactions outside of the exon 11 region. In this regard, several proteins including BARD1, CtIP, and BAP, have been shown to interact with human BRCA1 through the amino and carboxyl terminal regions of the protein (25, 28, 54, 58). In addition to these functions, however, it is interesting to speculate that $p92^{Brca1}$ may also have functions that are distinct from those of $p210^{Brca1}$.

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## FIGURE LEGENDS

**Figure 1. Detection of mouse Brca1 isoforms.** A) Immunoblot analysis demonstrating that mAb1, mAb2, and mAb4 recognize murine Brca1. 10 μg of either empty vector (pBKCMV) or pBKCMVBrca1 (mAb1) and pcDNA3.1 or pcDNA3.1-mBrca1 (mAb2 and mAb4) was introduced into 293T cells by calcium phosphate transfection. (-) indicates empty vector and (+) indicates vector containing murine *Brca1* cDNA. Cell extracts were prepared 48 hrs following transfection and 50 μgs of lysate was used for immunoblotting. Affinity purified antibodies were employed at 1 μg/ml. B) Northern analysis demonstrating the absence of full length *Brca1* transcripts in $Brca1^{\Delta 11/\Delta 11}$ MEFS. 10 μg of polyA mRNA was loaded per lane. Probes encompassing exon 11-specific sequences (right panel and Materials and Methods) and C-terminal nucleotides (left panel and Materials and Methods) were derived by PCR amplification using the mouse *Brca1* cDNA as a template. C) Immunoblot analysis of $p210^{Brca1}$ expression in wild-type and $Brca1^{\Delta 11/\Delta 11}$ MEFs. 50 μgrams of cell extract per lane was probed with affinity purified mAb1 at 1μg/ml. D) mAb1 recognizes a predominant gene product of ~ 92 kDa in $Brca1^{\Delta 11/\Delta 11}$ MEFs and 210 kDa full length Brca1 protein in HC11 cells. 30 μgrams of extract was loaded per lane. E) $p92^{Brca1}$ is expressed in testis and brain of wild-type mice. 150 μg of lysate per sample was subjected to PAGE on an 8% gel. Δ11/+Brain indicates tissue derived from a mouse heterozygous for the wild-type and exon 11-deleted alleles of *Brca1*.

**Figure 2. Mouse $p210^{Brca1}$ and $p92^{Brca1}$ are cell cycle regulated.** A) Immunoblot analysis of cell cycle regulation of $p210^{Brca1}$, Cyclin A, and Rad51 in serum-starved HCll cells. B) Immunoblot analysis of cell cycle regulation of $p92^{Brca1}$, Cyclin A, and Rad51 in serum-starved mouse embryonic fibroblasts homozygous for the targeted deletion of exon 11. Cells were starved as described in Materials and Methods. Cells stimulated to reenter the cell cycle by refeeding were harvested at the time points indicated. Active refers to exponentially growing cells. Cell extracts were prepared as described in Materials and Methods and 10 μg of lysate was loaded per lane. Antibodies mAb1 and

mAb2 revealed identical results in HC11 cells whereas only mAb1 recognized a cell cycle regulated band in *Brca1*$^{\Delta 11/\Delta 11}$ MEFS.

**Figure 3. p210$^{Brca1}$ but not p92$^{Brca1}$ undergoes a shift in response to DNA damage.** A) Immunoblot analysis of p210$^{Brca1}$ and p92$^{Brca1}$ in cells treated with UV, γ-irradiation, or HU. p210$^{Brca1}$ exhibits a dose-dependent shift in response to UV and gamma irradiation. HCll cells (top panel) or MEFs that express only p92$^{Brca1}$ (botton panel) were subject to identical treatments with UV, γ-irradiation, or HU. 20 μg of lysate was loaded per lane and immunoblotted with antibody mB1. B) Analysis of p210$^{Brca1}$ phosphorylation in $^{32}$P-orthophosphate labeled HCll cells treated with γ-irradiation (upper panel). Immediately following irradiation, HCll cells were incubated with 5 mCi of $^{32}$P-orthophosphate for 1 hr. 3 mg of cell extract was used for immunoprecipitation with 10 μl of the IgG fraction of mB1 antibody. The resolution of this assay was not sufficient to detect a mobility shift of phosphorylated products. Identical treatment of *Brca1*$^{\Delta 11/\Delta 11}$ MEFs irradiated with 20 Gy (lower panel).

**Figure 4. Localization of p210$^{Brca1}$ and p92$^{Brca1}$ to nuclear foci.** A) Western analysis of biochemical fractionation of *Brca1*$^{\Delta\text{-}11/\Delta\text{-}11}$ MEFs. Equal volumes of nuclear and cytoplasmic extract were loaded per lane. Antibodies were used as described in Materials and Methods. B) Schematic of murine *Brca1* cDNA indicating regions against which antisera were raised. Numbers above the lines represent amino acid coordinates. C) Immunofluorescence analysis of Brca1 subcellular localization. HCll cells, wild-type MEFs, and *Brca1*$^{\Delta 11/\Delta 11}$ MEFs were grown on microscope slides as described in Materials and Methods. Following permeabilization, S phase cells were incubated with affinity purified Brca1 antibodies at a concentration of 1 μg/ml.

**Figure 5. Rad51 association with p92$^{Brca1}$ is not detected in *Brca1*$^{\Delta 11/\Delta 11}$ MEFs.** Extracts generated from cycling HC11 and *Brca1*$^{\Delta 11/\Delta 11}$ MEFs were prepared as described in Materials and Methods. 1 mg of extract was used per sample for immunoprecipitation with 2 μg of antibody. mAb-

1 and mAb3 were affinity purified. Rad51 antibody was used at 1:1000 for Western analysis. Cross-reacting faint bands in mAb1, mAb3, and HA-Y11 lanes do not comigrate with Rad51.

**Figure 6. Impaired Rad51 foci formation in *Brca1*$^{\Delta 11/\Delta 11}$ MEFs.** A) A selection of representative Rad51 immunostained nuclei from WT and *Brca1*$^{\Delta 11/\Delta 11}$ MEFs three hours following irradiation with 10 Gy. Cells were prepared for immunofluorescence as described in Materials and Methods. Foci counts were obtained by visual inspection of 50 nuclei. B) Graph of numbers of foci per nuclei following irradiation with 5 and 10 Gy (p values = 1.28E-44 and 8.35E-51 respectively). C) Rad51 levels do not change in response to irradiation in WT and *Brca1*$^{\Delta 11/\Delta 11}$ MEFs. 3 hrs following irradiation with the indicated doses, extracts were prepared and analyzed by immunoblotting as described in Materials and Methods.

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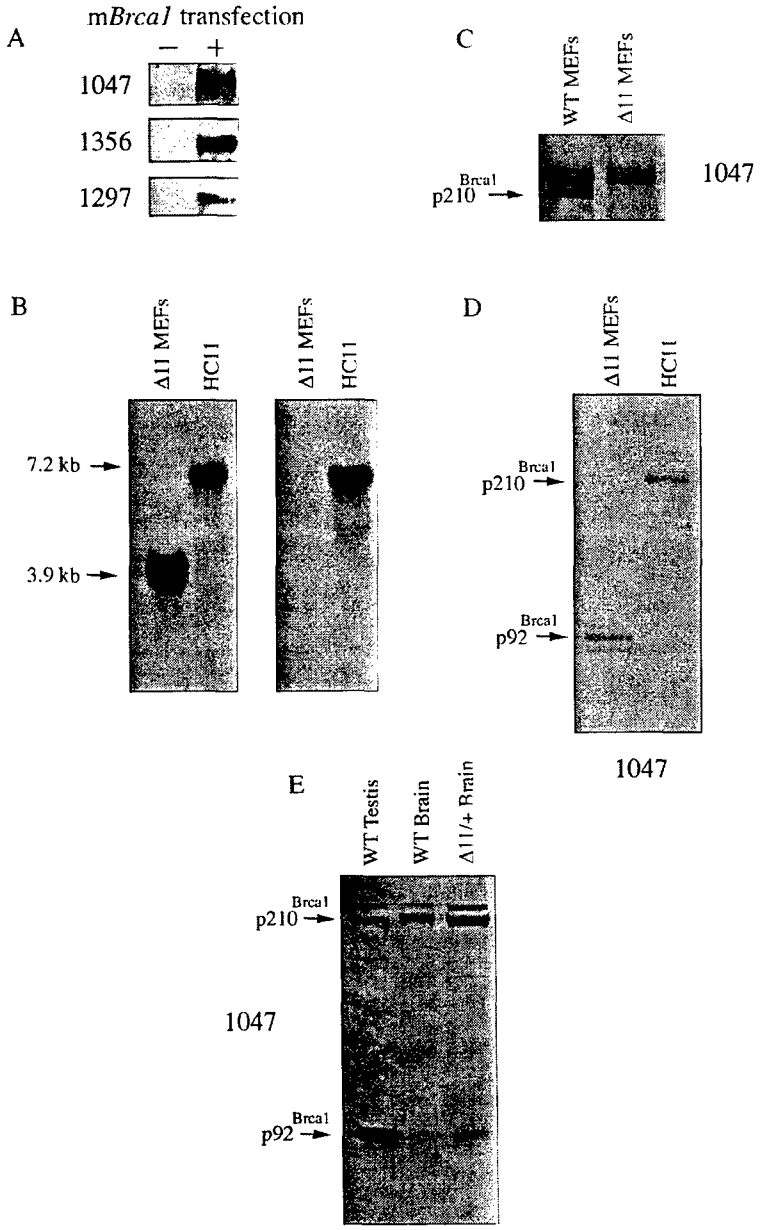
# FIGURE 1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C
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Δ11 MEFs
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1047
D
Δ11 MEFs
HC11
Brca1
p210
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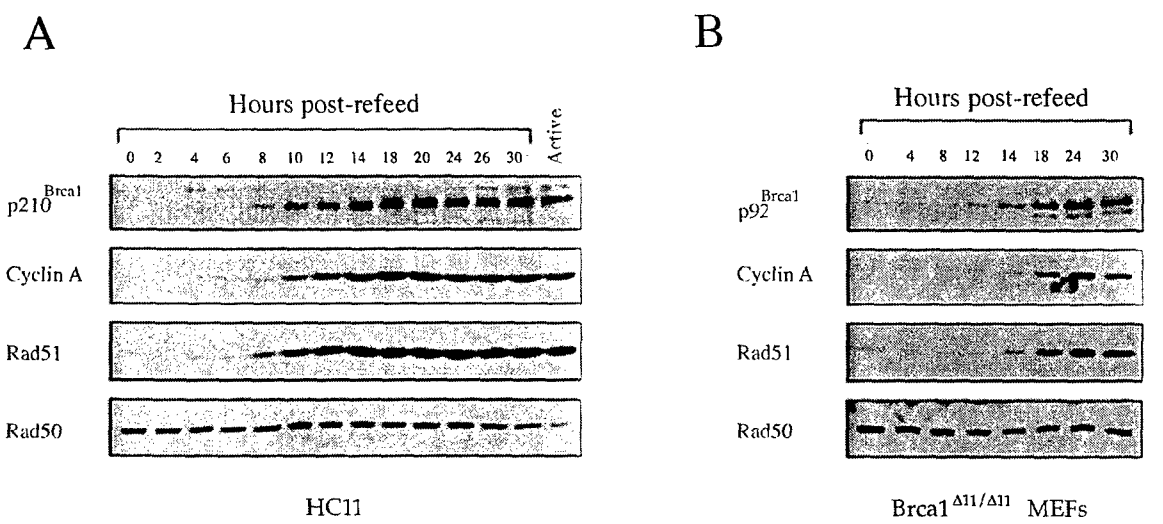
Brca1
p92

# FIGURE 2


A
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Active
p210 Brca1
Cyclin A
Rad51
Rad50
HC11
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0 4 8 12 14 18 24 30
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Cyclin A
Rad51
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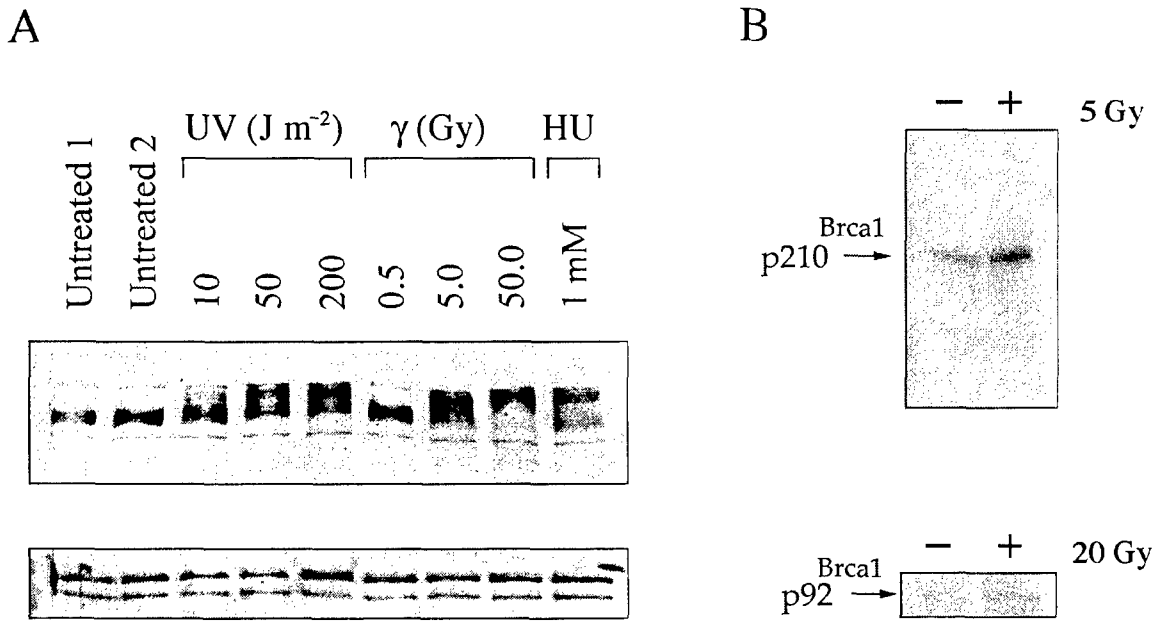
Brca1 Δ11/Δ11 MEFs

# FIGURE 3


A
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p210
– +
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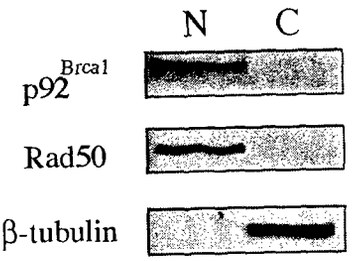
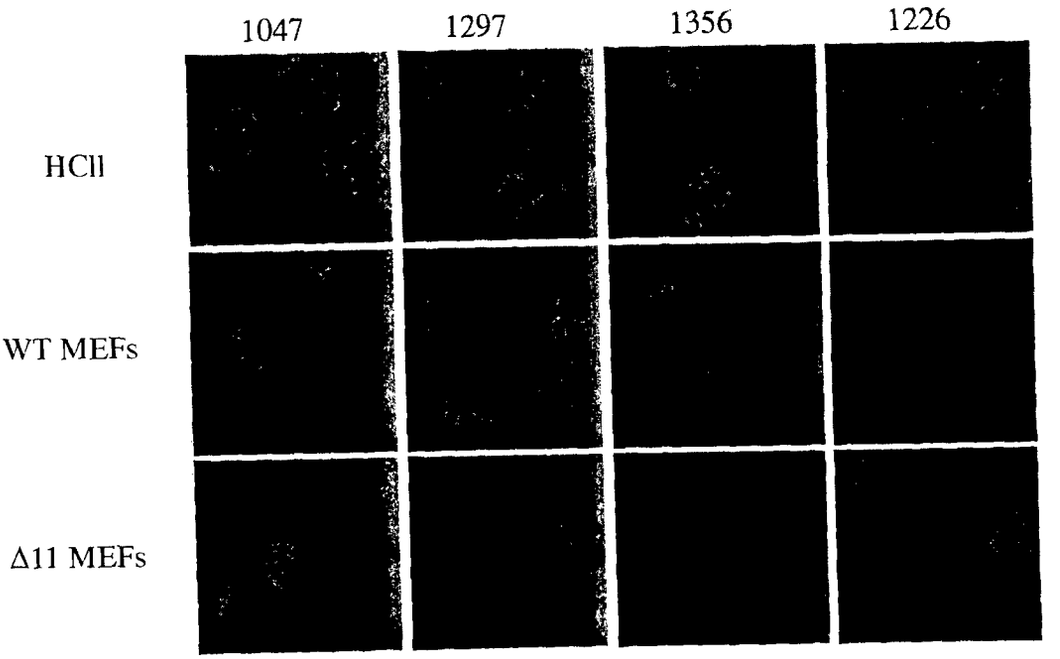
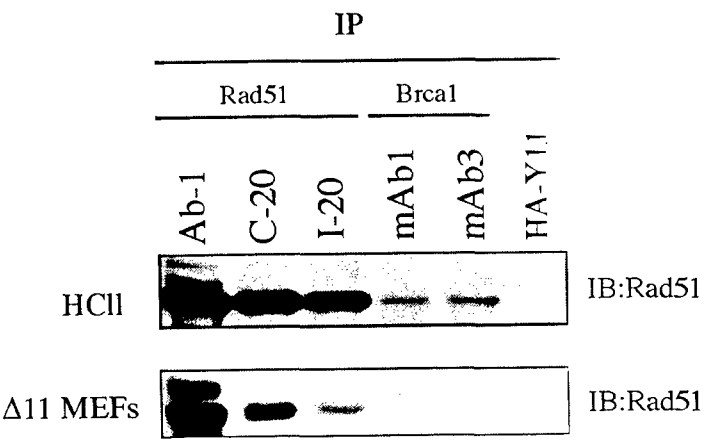
Brca1
p92

# FIGURE 4


N
C
p92 Brca1
Rad50
β-tubulin

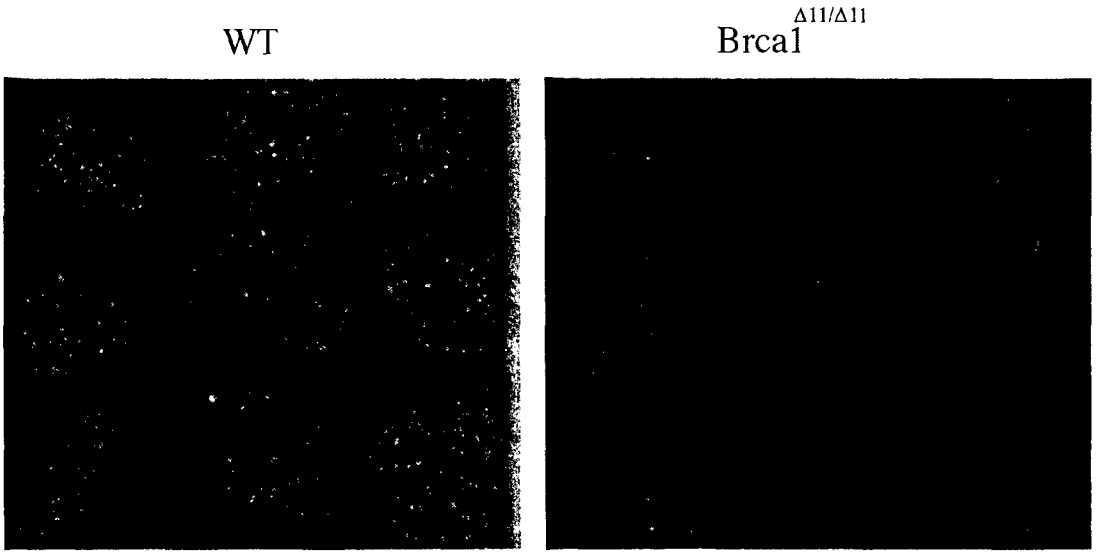

1047
1297
1356
1226
HCll
WT MEFs
Δ11 MEFs

# FIGURE 5

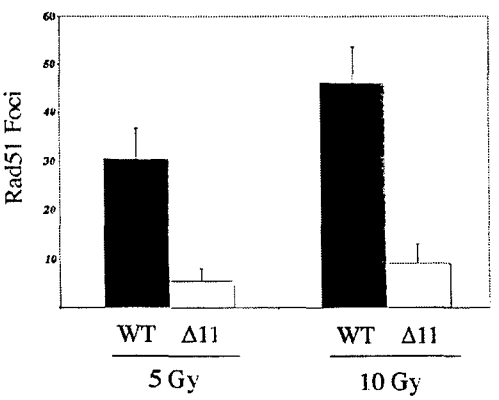

IP
Rad51
Brca1
Ab-1
C-20
I-20
mAb1
mAb3
HA-Y11
HCll
IB:Rad51
Δ11 MEFs
IB:Rad51

# FIGURE 6

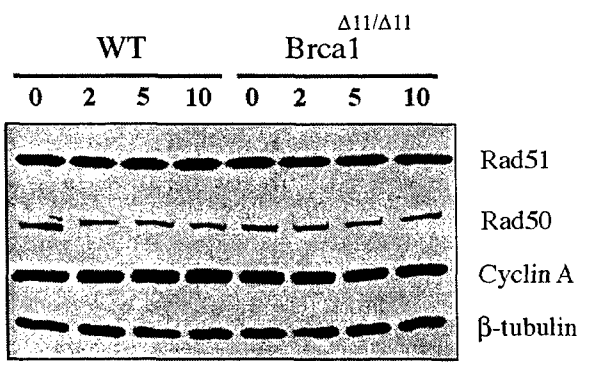
A


WT
Brca1 Δ11/Δ11

B


Rad51 Foci
60
50
40
30
20
10
WT Δ11
5 Gy
WT Δ11
10 Gy

C


WT
Brca1 Δ11/Δ11
0 2 5 10 0 2 5 10
Rad51
Rad50
Cyclin A
β-tubulin

# A Novel Doxycycline-Inducible System for Transgenic Analysis of Mammary Gland Biology

Edward J. Gunther[1,2], George K. Belka[1], Gerald B.W. Wertheim[1], James Wang[1], Jennifer L. Hartman[1], Robert B. Boxer[1], and Lewis A. Chodosh[1,3,*]

[1]Department of Molecular & Cellular Engineering
[2]Division of Hematology-Oncology
[3]Division of Endocrinology, Diabetes and Metabolism
University of Pennsylvania School of Medicine
Philadelphia, PA 19104-6160

*To whom correspondence should be addressed at 612 Biomedical Research Building II/III,
421 Curie Boulevard, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6160.
Tel: (215) 898-1321; Fax: (215) 573-6725; Email: chodosh@mail.med.upenn.edu

**Abstract:**

Reproductive endocrine events such as puberty, pregnancy and parity influence mammary glan susceptibility to tumorigenesis in both humans and rodents. Transgenic mouse models that rely on mammary-specific promoters to directly control transgene expression have limited utility for studying the impact of reproductive events on tumor risk since hormonal cues that govern these reproductive events also profoundly affect transgene expression levels. We have developed a mammary-specific, doxycycline-inducible transgenic mouse model for studying the impact of mammary gland developmental stage on transgene-mediated phenotypes. This system uses the MMTV-LTR to drive expression of the reverse tetracycline-dependent transactivator, *rtTA*, which can in turn control expression of an independently integrated, tet operator-controlled transgene. Unlike other mammary-specific, inducible transgenic systems that have been described, the system described here combines spatially homogeneous transgene expression in the mammary epithelium during puberty, pregnancy, lactation and involution with utilization of an orally administered, inexpensive, widely available induc Additional features of this system include a high degree of mammary epithelial cell-specific transgene expression, rapid kinetics of transgene induction and de-induction, undetectable transgene expression i the absence of inducer, and the ability to induce graded levels of transgene expression. This system offers new opportunities for transgenic analysis of mammary epithelium *in vivo*.

**Introduction:**

Epidemiologic and animal studies strongly suggest that the susceptibility of the mammary gland to carcinogenesis is a function of the gland's developmental state at the time of exposure to oncogenic stimuli. Specifically, reproductive endocrine events such as puberty, pregnancy, and parity each influence the susceptibility of the mammary gland to the subsequent development of cancer. At present, the molecular and cellular mechanisms by which normal developmental events modulate breast cancer risk are unknown. Understanding these mechanisms will undoubtedly require a more complete understanding of the interaction between mammary development, reproductive history, and oncogenic pathways than currently exists.

The postnatal development of the mouse mammary gland closely resembles that of the human and provides a unique and powerful model for studying the relationship between developmental biology and cancer. For example, several oncogenes implicated in human breast cancer have been overexpressed in the mammary glands of transgenic mice providing confirmation of both their carcinogenic potential and their ability to disrupt normal programs of epithelial differentiation. While constitutive mammary-specific transgenic models have proved valuable, the utility of these models for probing the impact of reproductive events on tumorigenesis has been limited by the characteristics of available mammary-specific promoters. For instance, the mouse mammary tumor virus (MMTV) and whey acidic protein (WAP) promoters are hormonally regulated and are therefore markedly upregulated during pregnancy and lactation. As a result, reproductive events in these models alter both transgene expression and cancer risk,

thereby prohibiting any meaningful analysis of the effect of reproductive events on cancer susceptibility.

Recently, transgenic mouse models have been designed for mammary-specific transgene expression under the control of exogenous inducers. In the first system of this type reported, the MMTV-LTR was used to drive expression of the tet-responsive transactivator, tTA, which activates expression of a second transgene controlled by tet operator sequences in the absence of tetracycline. This system has successfully been used to demonstrate the time-dependent reversibility of SV40 T antigen-induced salivary gland hyperplasias and BCR-ABL-induced leukemias in mice. However, this system may have limited usefulness for study of the mammary gland because of mosaic transgene expression; a small fraction of mammary epithelial cells demonstrate detectable reporter gene expression. Moreover, transgene induction in this model was detectable in a variety of tissues beyond the mammary gland. More recently, an MMTV-driven, inducible transgenic model utilizing a transactivator comprised of a modified ecdysone receptor was described. This model allowed homogeneous transgene expression in the mammary epithelium during lactation in the presence of the inducer, ponasterone A. Analysis of transgene expression during other stages of mammary gland development was not reported. Furthermore, unlike tetracycline-based systems, the system utilized an inducer that is not yet commercially available and requires parenteral administration.

We reasoned that application of transgenic mouse technology to analyses of the impact of development on breast cancer susceptibilty would require tightly regulated, mammary epithelial cell-specific transgene expression during any stage of post-natal mammary gland development. Ideally the system would permit homogeneous transgene

expression in the mammary epithelium, modulated transgene expression levels, and prompt kinetics of induction and de-induction. As described below, we have created a novel doxycycline-inducible mouse model system that we believe fulfills these criteria. In addition, this system permits early effects of trangene expression to be analyzed in normal epithelium. By permitting both the timing and level of transgene expression to be varied experimentally in a variety of developmental contexts, this model offers new opportunities for the study of differentiation and tumorigenesis in mammary epithelium *in vivo*.

## Materials and Methods:

**Transgenic Mice.** To create the plasmid pMMTV-*rtTA*-pA, the full length *rtTA* transgene (1.0 kb fragment from plasmid pUHD172-neo, a gift from Dr. Henry Bujard) was subcloned into pBluescript II KS (Stratagene) downstream of a 2.9 kb fragment containing the promoter elements derived from plasmid pMMTV-polyoma MT (a gift from Dr. Philip Leder). This promoter includes 1.2 kb of sequence upstream of the transcriptional start site of the MMTV-LTR and 0.6 kb of leader sequence from v-H-ras. 1.8 kb of SV40 sequence carrying splicing and polyadenylation signal sequences was subcloned downstream of the *rtTA* gene. The reporter construct pTetop-LacZ was created by subcloning the LacZ gene segment from plasmid pUHG16-3 (a gift from Dr. Bujard) downstream of a CMV minimal promoter and seven adjacent tet operator sites derived from pTet-Splice (Gibco). Again a 1.8 kb SV40 sequence was subcloned downstream of the coding sequence. The construct pTetop-Luc is identical to pTetop-LacZ except that the LacZ gene has been replaced by the firefly luciferase coding region excised from pGL3 (Promega).

Restriction fragments containing each transgene were isolated from vector sequences and prepared for microinjection into fertilized oocytes. All transgenic lines were created on an inbred FVB/N background. Potential founders were identified by screening genomic DNA from tail biopsies for the presence of the transgene using the polymerase chain reaction. Amplification reactions for genotyping animals used the following oligonucleotide pairs: MMTV-*rtTA*-pA: 5'-ATCCGCACCCTTGATGACTCCG-3' and 5'-GGCTATCAACCAACACACTGCCAC-3' to amplify a 349 bp segment spanning the

junction of MMTV-LTR and v-H-ras leader sequences; Tetop-LacZ: 5'-GGTCTGGACACCAGCAAGGAGCTGC-3' and 5'-GCGCATCGTAACCGTGCATCTGCC-3' to amplify a 307 bp sequence in the LacZ gene; Tetop-Luc, 5'- CACGAAATTGCTTCTGGTGGC -3' and 5'-TCGAAGATGTTGGGGTGTTGG-3' to amplify a 469 bp sequence in the luciferase gene. Reaction conditions were 40 cycles of 94 C for 30 seconds, 58 C for 30 seconds and 72 C for 30 seconds. Bitransgenic mice were derived from crosses between mice hemizygous for each transgene, except for the Tetop-LacZ founder line, TZA, which was bred to homozygosity and was used in the generation of some MTB/TZA and TZA mice. As observed for many transgenic mouse lines, male and female mice bred to be homozygous at the MTB locus were noted to be poor breeders. In addition, homozygous MTB dams often failed to raise their litters to weaning age. MTB heterozygote dams showed normal fertility and supported litter sizes typical of FVB/N mice.

Transgene expression in mice was induced by replacing their normal drinking water with 5% sucrose containing the indicated concentrations of doxycycline. For prolonged inductions, doxycycline-containing water was changed every 3 days. Mice were mated between 4 and 8 weeks of age. The day on which a vaginal plug was detected was defined as the first day of pregnancy. Pregnancy time points were confirmed by examination of the size and developmental stage of embryos at the time of sacrifice, and the first postpartum day was defined as day 1 of lactation. Regression time points were measured in days following the forced weaning of pups at day 12 of lactation.

**ß-galactosidase Solution Assay.** Harvested mouse mammary glands were snap frozen on dry ice and stored at –80 C. Protein extracts were prepared essentially as described (ref). Approximately 500 mg of frozen tissue was homogenized in 1.0 ml of buffer (40mM Tris-HCl [pH 7.4], 1 mM EDTA, 500 mM sucrose, 150 mM NaCl, 10mM dithiothreitol) using a polytron homogenizer. Homogenates were cleared by two centrifugation steps performed at 12,000 g for 20 minutes at 4 C. The soluble fraction was transferred to a fresh tube and protein concentration was quantitated by the method of Bradford. ONPG (o-nitrophenyl-B-D-galactopyranoside) was used as a colorimetric substrate in a standard ß-galactosidaseactosidase assay (ref). ß-galactosidase activity was measured by determination of a reaction rate. 10-30 micrograms of protein was assayed in replicate reactions that were terminated at increasing time points. The optical density of each reaction was measured at 420 nm and values were plotted against time to determine the reaction rate.

**X-gal Staining of Tissue Sections.** Mammary glands harvested for *in situ* determination of ß-galactosidase activity were frozen in OCT (Tissue Tek). Tissue blocks were stored at –80 C. Freshly cut tissue sections were applied to glass slides and pre-fixed in X% glutaraldehyde, then rinsed twice in PBS at room temperature for 20 minutes. Slides were then stained for ß-galactosidase activity at 37 C in an X-gal staining solution containing 1 mg/ml X-gal, 5mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 1mM $MgCl_2$. After staining, slides were rinsed twice for 20 minutes in PBS at room temperature, post-fixed in X% glutaraldehyde, rinsed in PBS, then coverslipped.

**Luciferase Assay.** Tissues were harvested and snap frozen on dry ice. Tissue hunks were homogenized in Passive Lysis Buffer (Promega) using a dounce homogenizer. Homogenates were cleared by centrifugation at 12,000 g, and the supernatant assayed for protein concentration by the method of Lowry and luciferase activity using the Dual Luciferase Assay Kit (Promega) and a Monolight 2010 luminometer (Analytical Luminescence Laborotory) according to manufacturers' instructions.

**Mammary Gland Histology.** Mammary glands were fixed in 4% paraformaldehyde overnight and embedded in paraffin. Sections were applied to glass slides and stained with hematoxylin and eosin.

## Results:

**Expression of rtTA in the mammary epithelium of transgenic mice.** Transgenic founder lines were generated harboring a construct, MMTV-*rtTA*-pA, in which expression of the reverse tetracycline-transactivator, rtTA ("Tet-On"), is driven by the MMTV-LTR. This construct contains a portion of the v-H-ras leader sequence, which has been associated with enhancd mammary expression of transgenes, downstream of the MMTV-LTR (ref). Although MMTV-promoter based transgenes are typically maximally expressed during late pregnancy and lactation, the MMTV-*rtTA* founder line, MTB, displayed easily detectable *rtTA* expression levels in the mammary glands of 5 week-old virgin female mice (Fig. 1A). Expression of *rtTA* appeared to vary little in age-matched virgin transgenic mice and, as expected, was unperturbed by the administration of doxycycline (Fig. 1A and data not shown).

**Inducible expression of reporter genes is tightly regulated and spatially homogeneous.** To determine the ability of the MTB line of mice to permit the inducible expression of transgenes, reporter mice were generated harboring the Tetop-LacZ transgene comprised of the bacterial LacZ gene downstream of a minimal promoter containing a heptamer of tet operator sequences. MTB mice were crossed with mice of the Tetop-LacZ-bearing transgenic line, TZA. Wild-type, MTB, TZA, and MTB/TZA bitransgenic nulliparous female mice were either induced by administering 2 mg/ml doxycycline in the drinking water for 72 hours or left untreated. Mammary gland-derived

protein extracts were then assayed for ß-galactosidase activity. As shown in Figure 1B, neither uninduced MTB/TZA glands nor induced MTB or TZA glands yielded ß-galactosidase activity above the background activity observed in wild-type FVB glands. In contrast, mammary extracts derived from doxycycline-treated MTB/TZA mice exhibited ß-galactosidase activity levels approximately 500-fold above background. These results indicate that reporter gene expression in this system is both highly inducible and tightly regulated.

Heterogeneous, or mosaic transgene expression in the mammary epithelium has frequently been observed in MMTV-based transgenic mouse models. As a result, transgene-mediated phenotypes may have low penetrance and may reflect effects of transgene expression on selected subsets of cells rather than on the epithelium as a whole. To determine the spatial localization of reporter gene expression in this system, *in situ* histochemical staining for ß-galactosidase activity was performed on whole-mounted mammary glands harvested from doxycycline-induced MTB/TZA bitransgenic mice. This analysis confirmed induction of ß-galactosidase activity in doxycycline-treated bitransgenic animals as well as the lack of detectable ß-galactosidase activity in uninduced bitransgenic animals. Furthermore, this analysis demonstrated that induction of ß-galactosidase activity in 6 week-old virgin female mice is confined to the mammary epithelial tree (Fig. 1C). Staining of frozen mammary sections from these mice further showed that ß-galactosidase activity in the mammary epithelium of induced MTB/TZA mice is remarkably homogeneous. Histochemical staining of sections failed to detect ß-galactosidase activity in uninduced MTB/TZA glands or monotransgenic genetic controls (Fig. 1C and data not shown).

**Transgene induction is mammary-specific.** Northern hybridization analysis of tissues derived from 6 week-old virgin MTB mice demonstrated a high degree of mammary-specific expression (Fig. 2A). Consistent with other MMTV transgenic models, lower levels of expression were observed in the salivary gland and the male seminal vesical (Fig. 2A and data not shown). These findings suggest that *rtTA* trangene expression in MTB mice may demonstrate a greater degree of mammary specificity than is typically seen in other MMTV transgenic models (ref).

To further investigate tissue specificity in this model system, transgenic mice carrying the luciferase gene under the control of tet-operator sequences (Tetop-Luc) were generated and crossed to MTB mice. Assays to detect luciferase gene expression are more sensitive and have a larger dynamic range than assays for ß-galactosidase activity. Bitransgenic MTB/TetopLuc mice were induced for 72 hours with 2 mg/ml doxycycline. Both genetic and uninduced controls were analyzed in parallel. Luciferase assays were performed on protein samples from a panel of 17 tissues and normalized to protein concentration. These studies demonstrate the induction of luciferase activity by more than three orders of magnitude in the mammary glands of MTB/Tetop-Luc bitransgenic mice, and somewhat lower levels of induction in the salivary glands of the animals (Fig. 2B). Low but detectable levels of expression were observed in the thymus, a tissue previously shown to express MMTV-driven transgenes. Notably, neither mammary gland, salivary gland, nor thymus demonstrated any detectable luciferase activity in the absence of doxycycline induction (Fig. 2B). These findings confirm that this bitransgenic system is both mammary-specific and tightly regulated by doxycycline.

**Titratable levels of transgene expression.** One of the principal advantages of an inducible expression system is that it permits the titration of transgene expression to a desired level. The ability to regulate transgene expression levels in the mammary epithelium is required for studying how the level of transgene expression impacts on mammary gland phenotype, and for achieving comparable levels of transgene expression during different developmental stages. Accordingly, the ability to reproducibly titrate transgene expression levels in the mammary gland was tested by generating a dose-response curve for reporter gene induction in MTB/TZA mice. Nulliparous female MTB/TZA bitransgenic mice were administered increasing doxycyline doses via drinking water for 72 hours prior to mammary gland harvest. Protein extracts prepared from these glands were analyzed for ß-galactosidase activity (Fig. 3A). Induction of ß-galactosidase activity in MTB/TZA mice was first detectable at a doxycycline concentration of 0.03 mg/ml and was near maximal at 0.5 mg/ml. Intermediate doses of doxycycline reproducibly induced intermediate levels of ß-galactosidase activity. These data demonstrate that this doxycycline-dependent transgenic system permits transgene expression to be titrated to a desired level. The fact that steady-state levels of transgene expression are dependent upon the half-lives of the specific mRNA and protein studied, however, will necessitate that similar studies be performed for each bitransgenic model in which expression levels will be titrated.

**Rapid induction of transgene expression.** The ability to determine the short-term effects of transgene induction on normal tissues requires the ability to rapidly induce

transgenes. Moreover, precise timing of transgene induction is critical for studying the impact of transgene expression on developmental processes. Accordingly, the kinetics of LacZ transgene induction in MTB/TZA bitransgenic mice was determined by measuring ß-galactosidase activity in the mammary glands of nulliparous females following exposure to doxycycline. As observed previously, no ß-galactosidase activity was detected in uninduced MTB/TZA glands above the background activity detected in wild-type glands. In contrast, ß-galactosidase activity was first detected in MTB/TZA animals 6 hours after doxycycline exposure (Fig. 3B). ß-galactosidase activity continued to increase with increasing times of doxycycline exposure up to 1 week, most likely as a consequence of the long half-life of the LacZ mRNA transcript and encoded ß-galactosidase protein. In contarst, MTB mice bitransgenic for an inducible c-MYC transgene attain steady-state levels of c-MYC expression within 48 hours of induction, presumably reflecting the short half-life of the c-MYC mRNA and protein (ref and data not shown).

The ability to turn off transgene expression is also a desirable property of inducible systems. The kinetics of transgene deinduction would be expected to depend on both the half-life of the transgene product and the rate at which doxycycline levels decline *in vivo* after discontinuing the drug. Accordingly, analysis of MTB/Tetop-MYC mice demonstrates that c-MYC transgene expression levels decline to baseline levels within 24 hours following doxycycline withdrawal. (ref. and data not shown).

**Homogeneous reporter gene expression during multiple stages of mammary development.** To characterize transgene induction at other stages of postnatal mammary

development, ß-galactosidase activity was analyzed in the mammary glands of MTB/TZA bitransgenic female mice at developmental stages representing puberty, pregnancy, lactation, and post-lactational-involution. MTB/TZA females and genetic controls were induced with 2 mg/ml doxycycline for 72 hours prior to mammary gland harvest. ß-galactosidase activity was highly induced at each developmental time point analyzed (Fig. 4A). As expected, the levels of ß-galactosidase activity detected in the pregnant and lactating glands of MTB/TZA mice exceeded the levels detected in virgin glands (Fig. 4A). This is consistent with the finding that *rtTA* expression is higher during pregnancy and lactation. (data not shown).

A major limitation of mammary-specific transgenic models, particularly those utilizing the MMTV-LTR, has been marked spatial heterogeneity of transgene expression in the mammary epithelium. While our initial data indicated that transgene expression is relatively homogeneous in the mammary glands of 6 week-old mice, we wished to characterize transgene expression at other stages of postnatal development.
The spatial distribution of ß-galactosidase activity was analyzed at the cellular level by histochemical staining of mammary gland frozen sections. Homogeneous staining throughout the mammary epithelium was observed in glands harvested from induced MTB/TZA bitransgenic females during puberty, pregnancy, lactation and post-lactational involution (Fig. 4B).

Due to the fact that the epithelium comprises a higher fraction of cells within the mammary gland of pregnant as compared to virgin mice, direct comparison of ß-galactosidase activity in homogenates of mammary glands from MTB/TZA mice at different developmental stages can be problematic even when normalizing activity to

protein levels. In this regard, it is worth noting that sections from pregnant and lactating induced bitransgenic glands exhibited more intense staining for ß-galactosidase activity than comparable sections from virgin mice analyzed in parallel. This suggests that induced ß-galactosidase activity is greater on a per-cell basis in mammary epithelial cells of pregnant and lactating MTB/TZA mice compared to virgin MTB/TZA mice.

**Heterogeneous transgene expression in aging mice.** The absolute levels of LacZ transgene expression was observed to decrease in MTB/TZA bitransgenic mammary glands harvested from 15 weeek-old as compared to 6 week-old mice (Fig. 5). Consistent with this, expression of *rtTA* in the mammary gland of MTB mice also decreases with age (data not shown). To determine the basis for this change, 15 week-old nulliparous and parous MTB/TZA mice were analyzed for transgene expression by histochemical staining of mammary gland sections. Unlike other stages of mammary gland development that were analyzed, staining for ß-galactosidase activity was heterogeneous in these groups of bitransgenic mice (Fig. 5). As such, the observed decrease in reporter transgene expression in these mice may, in part, be a consequence of expressing the transgene in only a portion of mammary epithelial cells. At present, it is unclear whether unstained cells fail to express the transgene or express the transgene at a level below the limits of histochemical detection.

**Postnatal mammary gland development appears normal in MTB transgenic mice.** In order for the MTB bitransgenic system to be of maximum utility, mammary development in these mice must be demonstrably normal. This criterion is particularly

important given concerns that overexpression of a strong transcriptional transactivator, such as rtTA, may be toxic to mammary epithelial cells. To address this issue, mammary glands from MTB and wild-type mice were analyzed morphologically for evidence of developmental abnormalities by examination of carmine-stained whole mounts and hematoxylin and eosin-stained sections (Fig. 6). The highly ordered nature of the mammary epithelial tree permits this type of analysis to detect relatively subtle changes in gland development. These studies demonstrate that at the morphological and histological levels, mammary development in MTB animals is indistinguishable from that observed in wild-type mice at each stage of postnatal development, including puberty, pregnacy, lactation and postlactational involution.

To determine whether high levels of rtTA expression alters mammary epithelial proliferation, BrdU incorporation in the mammary epithelium was measured during puberty in MTB mice. Levels of BrdU incorporation were indistinguishable between cohorts of MTB and wild-type age-matched virgin female mice (data not shown). In addition, litter number, litter size, and the ability to rear pups was indistinguishable when comparing MTB hemizygous and FVB/N wild-type dams (data not shown). These findings suggest that mammary development is normal in MTB hemizygous mice.

**Discussion:**

This report describes a mouse model that displays many ideal features of an inducible, mammary-specific expression system including: 1) undetectable reporter transgene expression in the uninduced state, 2) titratable induced expression levels over a broad range with relatively little variation in expression among animals of similar age and reproductive history, 3) inducible transgene expression that is spatially homogeneous in the mammary epithelium when analyzed during puberty, pregnancy, lactation or involution, 4) prompt kinetics of induction and de-induction, and 5) utilization of an inexpensive, widely available, easily administered, and essentially inert inducer. Analysis of our model system provides the first demonstration of homogeneous, inducible transgene expression in the mammary epithelium of nulliparous mice. Inasmuchas this system was designed for studying the impact of reproductive events on transgene-mediated phenotypes in the mammary epithelium, the ability to induce homogeneous transgene expression in a variety of developmental settings is critical. Mosaic transgene expression in the mammary epithelium may lead to low penetrance of transgene-mediated phenotypes and may restrict transgenic analysis to an undefined subset of epithelial cells.

A number of novel experimental approaches to transgenic analysis of the mammary epithelium are facilitated by inducible control over transgene expression. Our recent application of the MTB transgenic line described in this report to inducible expression of a c-MYC transgene highlights the experimental opportunities that stem

from the ability to turn off transgene expression. We crossed the MTB transactivator-bearing transgenic line described in this report to a second transgenic mouse line carrying a c-MYC transgene under the control of tet operator sequences. Analysis of bitransgenic mice showed that mouse mammary gland tumors induced by expression of the c-MYC transgene frequently remain dependent on continued transgene expression for tumor maintenance. Inducible expression of the c-MYC oncogene using this model system resulted in the formation of invasive mammary adenocarcinomas in a manner that was rapid, highly penetrant, mammary-specific, and absolutely dependent on transgene induction by doxycycline. In the absence of doxycycline induction, c-MYC transgene expression is undetectable and uninduced bitransgenic animals display a normal mammary phenotype. Interestingly, nearly 50% of tumors induced by c-MYC were found to carry activating mutations in K-ras or N-ras, and the presence of such mutations correlated with the acquired ability of these MYC-induced tumors to grow in a MYC-independent manner.

In addition, the model system described here permits modulation of transgene expression levels, which will facilitate analysis of the impact of transgene expression levels on phenotype. Amplification and overexpression of oncogenes, such as c-Myc and erbB2, is found in a subset of human breast cancers, yet little is known about the dose-response relationship between oncogene expression levels and mammary epithelial cell phenotype. Application of our model system to inducible expression of gain-of-function oncogenes will permit analysis of the consequences of graded levels of oncogene expression in the mammary epithelium of genetically identical mice without need for hormonal manipulation. Tightly regulated temporal control over transgene expression

allows oncogene expression to be initiated in normal epithelium of adult mice. Moreover, oncogene overexpression can be restricted to any stage of post natal mammary gland development permitting analysis of the impact of reproductive events on oncogene-mediated phenotypes.

Finally, strategies using inducible expression of the cre recombinase appear promising for tissue-specific, conditional gene targeting. Doxycycline-inducible cre expression has been used to conditionally delete loxP-flanked gene segments in mouse mammary epithelium. Use of the WAP promoter to drive *rtTA* expression in this model resulted in the requirement that gene deletion take place during lactation. Even under optimal conditions, cre-mediated recombination occurred in a small minority of epithelial cells. Mosaic gene deletion may be desirable for some experiments, but disadvantageous for others, especially those designed to study the impact of gene deletion on development. Experiments are currently underway to examine whether the MTB transgenic line will permit cre-mediated deletion in the mammary epithelium to be more homogeneous and less dependent on hormones of pregnancy.

Figure Legends

Figure 1. Transgene expression in MTB/TZA bitransgenic mice. (A) Reproducible expression of rtTA in the mammary glands of MMTV-rtTA-pA (MTB) mice. Northern hybridization analysis of total RNA from the mammary glands of six week-old nulliparous transgenic and wild-type female mice for rtTA expression. The presence or absence of the transgene is as indicated. Mice were administered 2 mg/ml doxycycline in drinking water for 72 hours as indicated. Mammary-specific expression of rtTA in MTB mice. Northern hybridization analysis of total RNA from the indicated tissues harvested from 6 week-old virgin MTB mice for rtTA expression. (B) Doxycycline-inducible expression of a B-galactosidase reporter gene in bitransgenic mice. Six week-old virgin female mice of the indicated genotypes were either left untreated or administered 2 mg/ml doxycycline in drinking water for 72 hours. Mammary gland extracts were prepared and assayed for B-galactosidase activity. (C) B-galactosidase activity is homogeneous and confined to the mammary epithelium of doxycycline induced MTB/TZA mice. Six week-old virgin female bitransgenic mice were either left untreated or were induced with doxycycline as above. Mammary glands were whole mounted (upper panels) or embedded in OCT

and sectioned (lower panels) prior to histochemical staining for B-galactosidase activity.

Figure 2. Mammary-specific transgene induction in bitransgenic mice. (A) Mammary-specific expression of rtTA in MTB mice. Northern hybridization analysis of total RNA from the indicated tissues harvested from 6 week-old virgin MTB mice for rtTA expression. (B) Doxycycline-inducible expression of a luciferase reporter in tissues from bitransgenic mice. A panel of 17 tissues were harvested from uninduced and induced 6 week-old nulliparous female mice bitransgenic for MTB and tetop-Luc. Protein extracts from the indicated tissues were analyzed for luciferase activity and compared with values obtained from wild-type littermates. Mice were induced with 2 mg/ml doxycycline administered in drinking water for 72 hours prior to tissue harvest.

Figure 3. Dose-response and kinetics of transgene induction. (A) Dose-response curve for doxycycline-inducible reporter gene expression. 6 week-old nulliparous female MTB/TZA bitransgenic mice and monotransgenic controls were administered the indicated doses of doxycycline in drinking water supplemented with 5% sucrose for 72 hours prior to tissue harvest.

Mammary gland protein extracts were prepared and assayed for B-galactosidase activity. (B) Kinetics of doxycycline-inducible reporter gene expression. 6 week-old nulliparous female MTB/TZA bitransgenic mice and monotransgenic controls were administered 2 mg/ml doxycycline in drinking water for the indicated times prior to tissue harvest. Mammary gland protein extracts were prepared and assayed for B-galactosidase activity, which is plotted as the percentage of that detected in MTB/TZA mice after one week of induction.

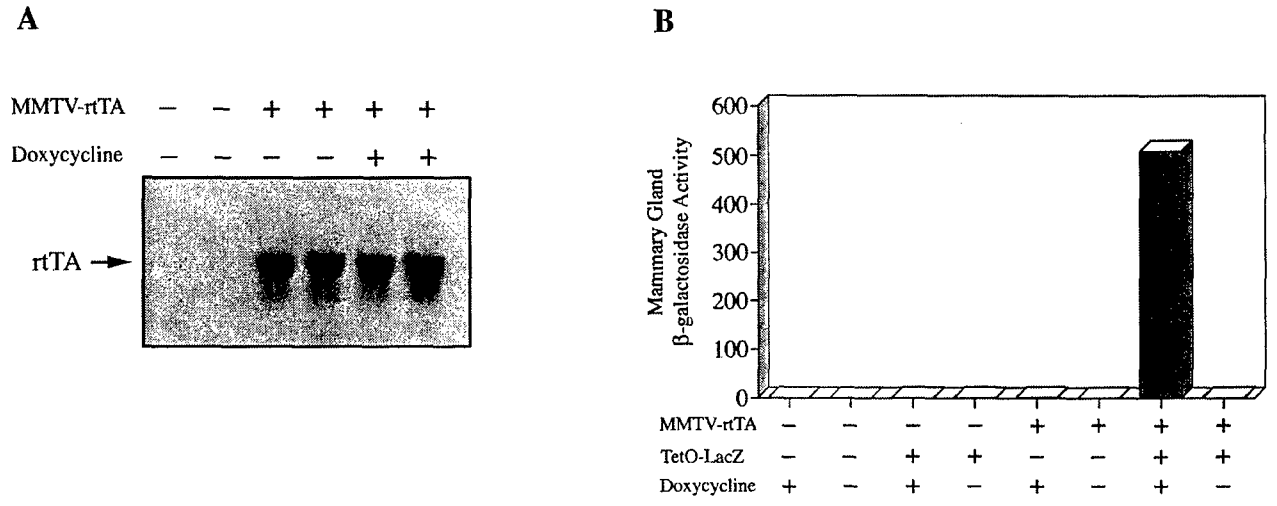
Figure 4. Developmental-dependence of doxycycline-inducible reporter gene expression. Bitransgenic MTB/TZA mice or monotransgenic TZA mice were either left untreated or induced with 2 mg/ml doxycycline drinking water for 72 hours prior to tissue harvest as above. (A) B-galactosidase activity measured in mammary gland protein extracts prepared from mice of the indicated genotypes and doxycycline exposure. The natural logarithm of these values is shown for 6 week-old and 15 week-old nulliparous (G0P0) mice, mice at day 6, 12, and 18 of pregnancy (G1P0), day 9 of lactation (LACT), and day 2 and 28 of post-lactational regression (REG). (B) Frozen sections of OCT-embedded mammary glands of the

induced and uninduced bitransgenic mice above were histochemically stained for B-galactosidase activity using X-gal.

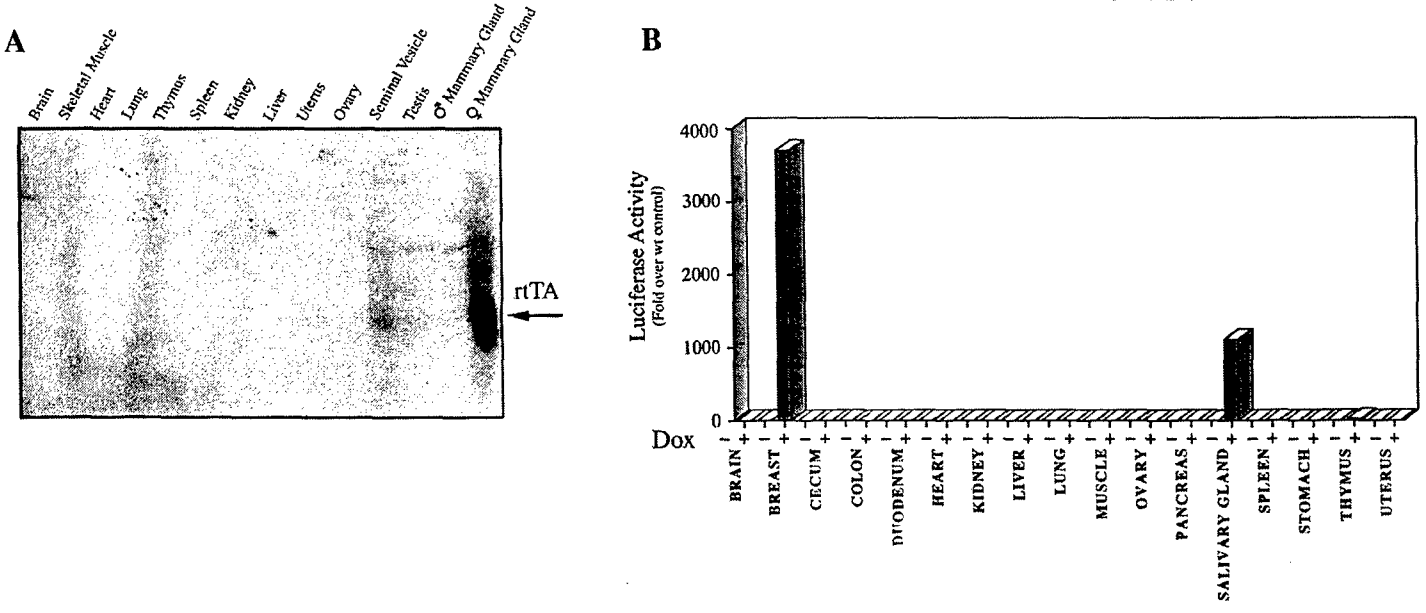
Figure 5. Heterogeneous reporter gene expression in mammary glands of 15 week-old mice. Frozen sections from OCT-embedded mammary glands from uninduced and doxycycline-induced nulliparous and parous 15 week-old bitransgenic MTB/TZA females were histochemically stained for B-galactosidase activity using X-gal. Doxycycline-induced mice were administered 2 mg/ml doxycycline in 5% sucrose as drinking water for 72 hours prior to tissue harvest.

Figure 6. Mammary gland morphology in MTB mice during development. Mammary glands were harvested from MTB transgenic and wild-type female mice at the indicated developmental time points. For analysis of whole mount morphology (A), inguinal mammary glands were spread on slides, fixed, and subjected to carmine staining of the epithelial tree (Ref.). For analysis of tissue histology, hematoxylin and eosin-stained sections (B) from paraffin-embedded mammary glands were analyzed by light microscopy at X magnification.

# FIGURE 1




# FIGURE 2



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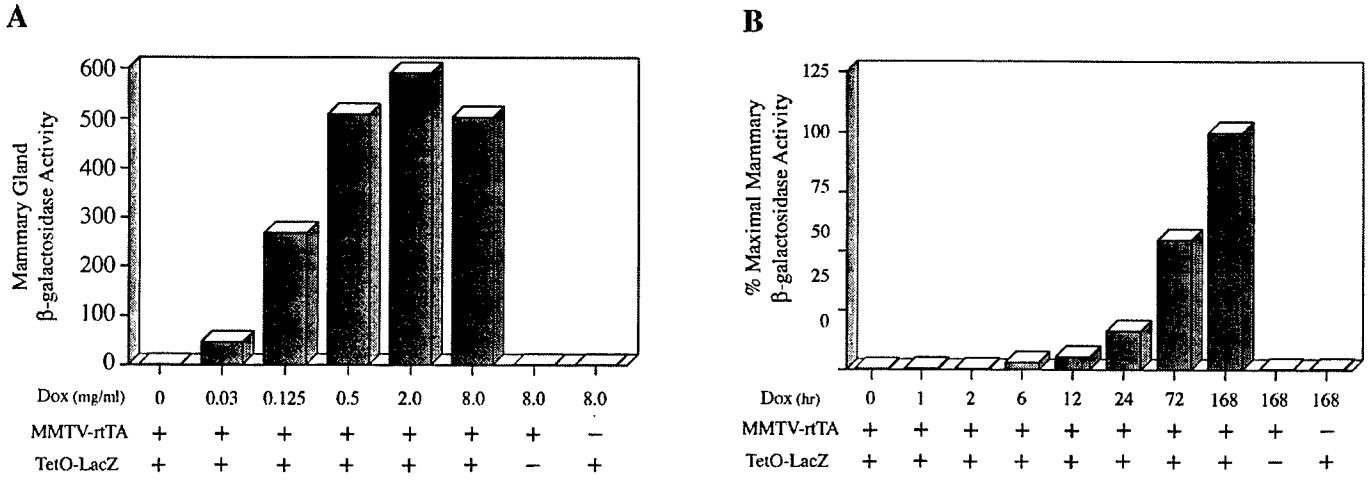
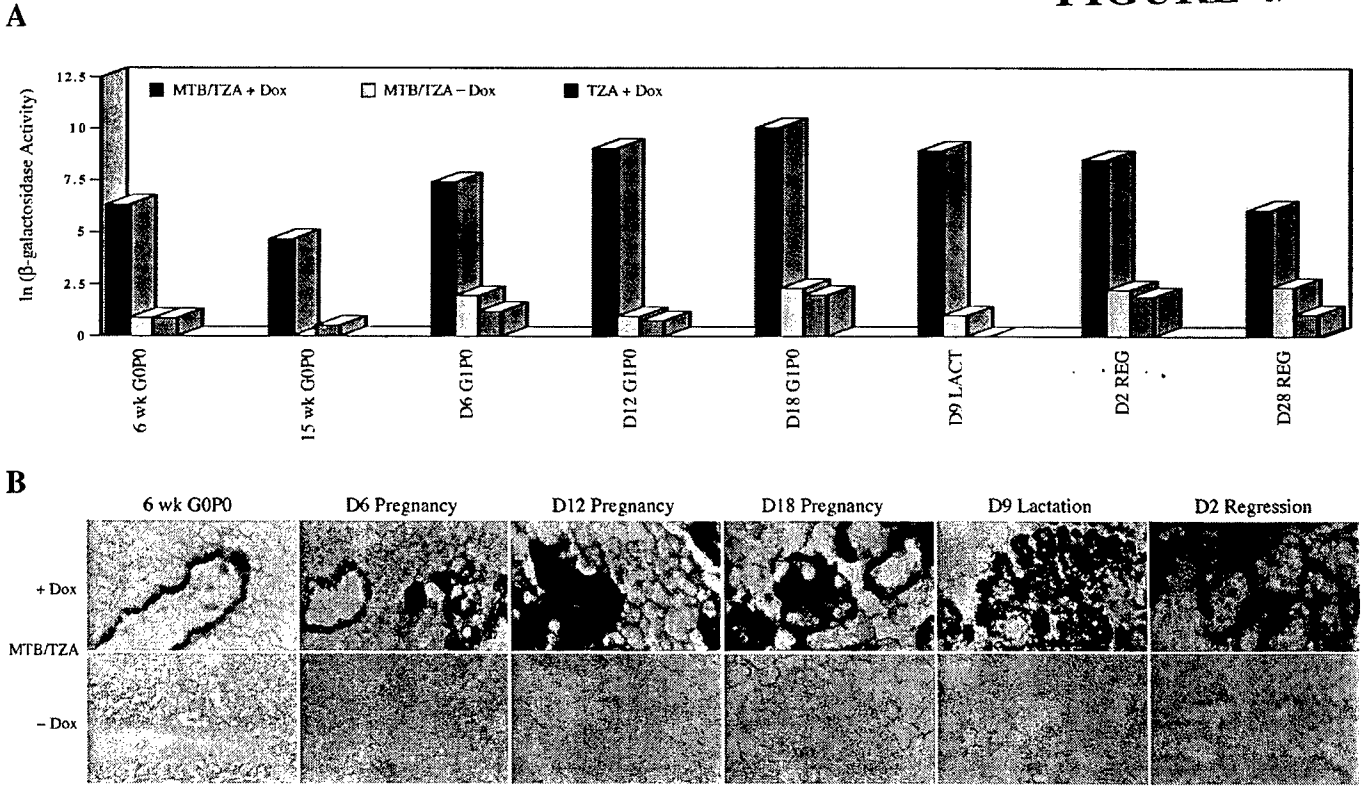



# FIGURE 4



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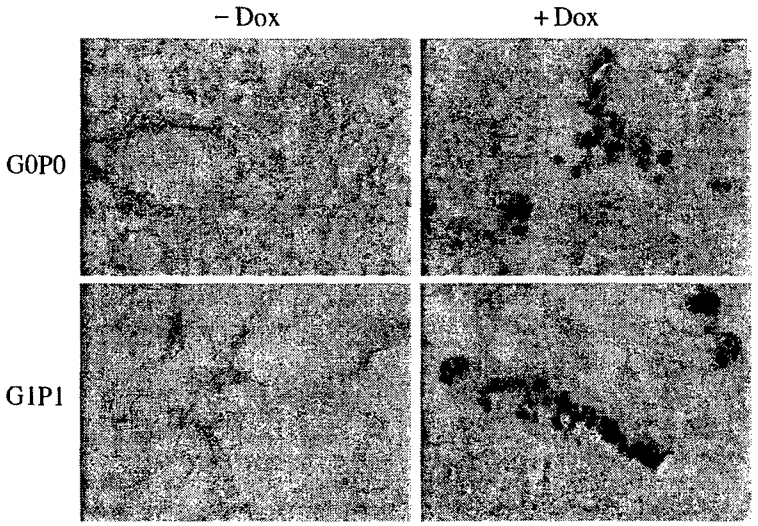


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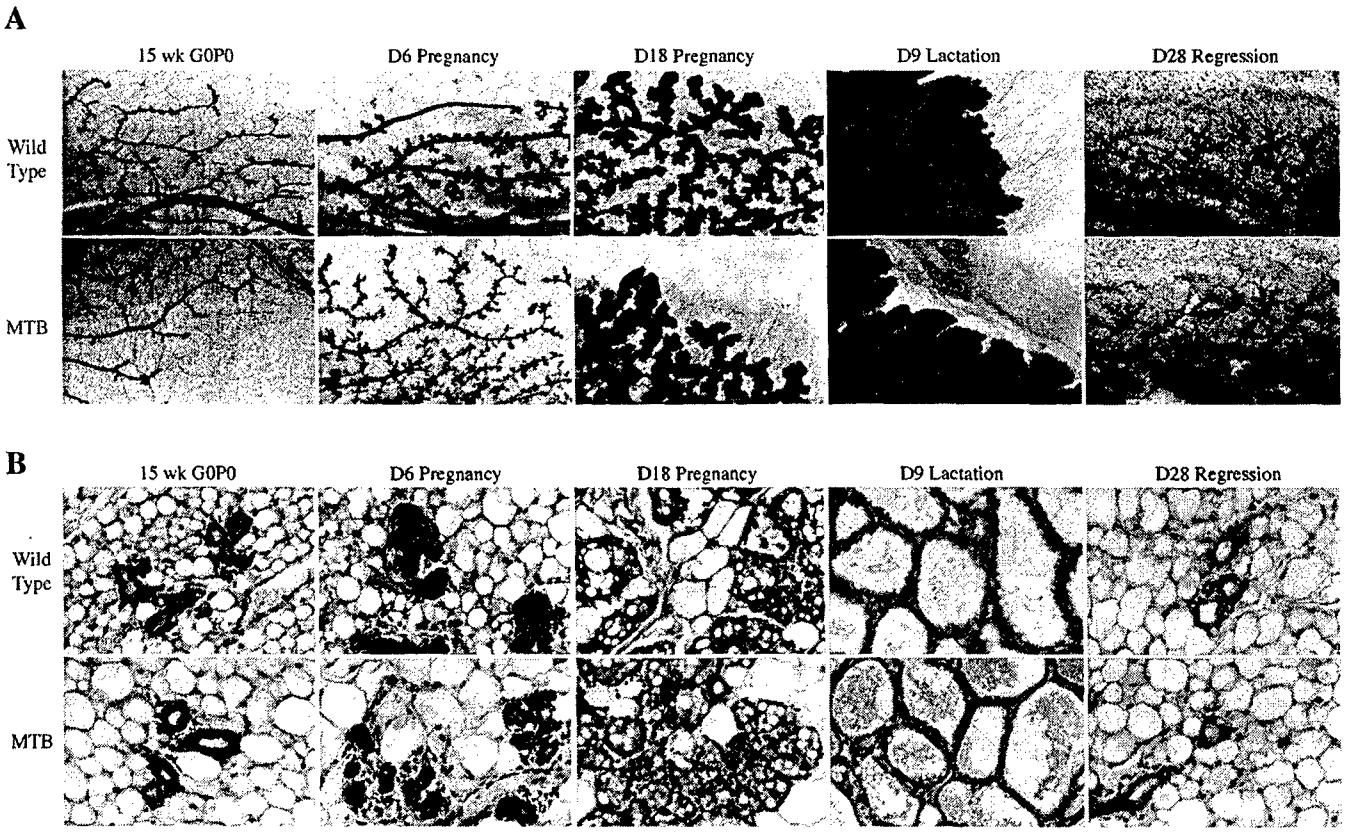

**DEPARTMENT OF THE ARMY**
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

Received 7/23/2001

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

Encl